SYNTHESIS, CHARACTERIZATION AND EVALUATION FOR ANTIMICROBIAL, ANTICANCER AND ANTIOXIDANT ACTIVITY OF SOME HETEROSTEROIDS

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

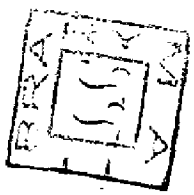
Doctor of Philosophy

IN

CHEMISTRY

BY

AYAZ MAHMOOD DAR



Under the supervision of

DR. SHAMSUZZAMAN

DEPARTMENT OF CHEMISTRY
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

2014

THESIS

THESIS

T-9091

16 NOV 2014

T9091

CANDIDATE'S DECLARATION

I, **Ayaz Mahmood Dar,** Department of **Chemistry** certify that the work embodied in this Ph.D thesis is my own bonafide work carried out by me under the supervision of **Dr. Shamsuzzaman** at Aligarh Muslim University, Aligarh. The matter embodied in this Ph.D thesis has not been submitted for the award of any other degree.

I declare that I have faithfully acknowledged, given credit to and referred to the research workers wherever their works have been cited in the text and the body of the thesis. I further certify that I have not willfully lifted up some other's work, para, text, data, result, etc. reported in the journals, books, magazines, reports, dissertations, theses, etc., or available at web-sites and included them in this Ph.D. thesis and cited as my own work.

Date: Jan. 10, 2014

Ayaz Mahmood

(Signature of the candidate)

Ayaz Mahmood Dar
(Name of the candidate)

Certificate from the Supervisor

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

Signature of the Supervisor:

10.1.2014

Name & Designation: **Dr. Shamsuzzaman**
(Associate Professor)

Department: **Chemistry**

(Signature of the Chairman of the Department with seal)

Chairman
Department of Chemistry
A.M.U., Aligarh 11/1/14

THESIS

COURSE/ COMPREHENSIVE EXAMINATION/ PRE-SUBMISSION SEMINAR COMPLETION CERTIFICATE

This is to certify that Mr. **Ayaz Mahmood Dar**, Department of **CHEMISTRY** has satisfactory completed the course work/comprehensive examination and pre-submission seminar requirement which is part of his Ph.D programme.

(Sig. of Supervisor)

(Signature of the Chairman of the Department)

Chairman
Department of Chemistry
A.M.U., Aligarh 11/1/14

Date: 10.1.2014

COPYRIGHT TRANSFER CERTIFICATE

Title of the Thesis: *Synthesis characterization and evaluation for antimicrobial, anticancer and antioxidant activity of some heterosteroids*

Candidate's Name: *Ayaz Mahmood Dar*

Copyright Transfer

The undersigned hereby assigns to the Aligarh Muslim University, Aligarh, copyright that may exist in and for the above thesis submitted for the award of Ph.D degree.

Ayaz Mahmood

(Signature of the Candidate)

DEDICATED

TO

MY PARENTS

Acknowledgement

Acknowledgement

At the beginning of my doctoral research, I could barely foresee how much I was to mature and gain intellectually in the years to come. A lot many people are responsible for this development; unfortunately, I am able to thank only a few here.

Foremost of them all is my Allah, the Almighty, the lord of all worlds, the most merciful, the most gracious, be witness, I acknowledge your every minute and greatest blessing on me, apparent and hidden favors, from the time before, to the time after this life, in all the quarters, in all the quarters including my research and writing of this Ph. D thesis, just for my belief in you.

I avail this unique opportunity to express my gratitude and indebtedness to my supervisor Dr. Shamsuzzaman. It is not justified if I simply thank him for steering me through the journey that has culminated in this thesis, giving me ample freedom to pursue my topic of interest, patiently reviewing my papers, providing timely and insightful comments and very generously allowing me to attend number of workshops and conferences. I have come to realize that the best way to express my gratitude for the support provided by him and for the role model he has been, is through continued contributions to organic chemistry – I am hopeful of doing so. Thank you Sir. I owe a great debt of gratitude to Prof. M. Mushfiq, Dr. Abdul Rauf, Dr. Mehtab Parveen, Prof. Sartaj Tabassum and Dr. Manzoor A. Gatoo for the invaluable suggestions and guidance during my Ph.D. Thank you all. I am also very thankful to Altaf Husain, Yusuf Khan, Zahid Yaseen, Mahboob alam, Aamir Sohail, Sheeraz Bhat, Imtiyaz Bhat, Mehvash Zaki, Suboohi Sherwani, Faisal Mustafa and Irshad Bhat for inviting me for fruitful collaboration and giving me advices from time to time and boost my morale. Needless to say, I look forward to many more such fruitful collaborations. I would also like to thank the anonymous referees of my papers for their constructive comments.

I am highly grateful to Chairman Department of Chemistry, AMU Aligarh for necessary research facilities. I am highly thankful to ICGEB New Delhi, IIIM Jammu, CDFD Hyderabad, Department of Biochemistry AMU Aligarh and Department of Medical Biochemistry JNMC AMU Aligarh for biological studies and UGC for financial support for the successful completion of my research.

As a Ph. D student, I spent most of my time in my lab. I would like to thank my fellow lab mates. Khursheed, Hena, Ashraf, Asif and Abad, you have all been my great friends. I am also thankful to all my other seniors like Dr. Tabassum, Dr. Gulfam Alam, Dr. Sultanat Dr. Rakshanda and Dr. Zoya for

their kind support and cooperation. Besides research activities, I also enjoyed my stay at residential Hostel and this time wouldn't have been fun, had it not been with people like Faheem, Nadeem, Mohsin, Javid, Jameel, Amin, Rather, Hilal, Rayees and Tariq.

I am at a loss for words to thank my parents for being the best people I know. Had it not been your prayers and patience, I would have been lesser than this. Thank you so much. My sister (Mrs. Irshada) and brother (Mr. Tanveer) has been an ultimate source of moral support. I hope they will enjoy reading this thesis. It is a pleasure to thank many of my closer ones back home M. Ramzan, Fazzia, Parvaiz Ahmad, Maheen, Adeeba, Shahid, Tawfeeqa, Rozey, Ajmal, Asmat, Muskaan, Urooj, Raheela, Saima, Aamir, Mamma G, Pyare G, Khala G, M.Isaaq Rather, Uncle, M. Yusuf, Sartaj, Summi, Gowhar, Mubarak Ahmad, shubli, Ashraf and Aante G whom I miss every time. Again, since there are too many of them, I am sorry at my inability to provide names here. My sincere apologies are to those whose names have been left out due to constraints on time and memory.

I am indeed thankful to Urfi Ishrat for their constant encouragement, emotional support and valuable suggestions.

"C mankind; we created you from a male and female and made you into nations and tribes that you may know and honour each other (not that you despise one another). Indeed the most honorable of you in the sight of God is the most righteous"

Al-Quran

Ayaz Mahmood Dar

TABLE OF CONTENT

Summary	I-VIII
List of publications	IX
Introduction	1

CHAPTER 1: Synthesis of steroidal pyrimidines

- Theoretical 2-13
- Discussion 14-22
- Experimental 23-28
- References 29-31

CHAPTER 2: Synthesis of steroidal pyrans

- Theoretical 32-40
- Discussion 41-49
- Experimental 50-53
- References 54-55

CHAPTER 3: Synthesis of steroidal pyrazoles and pyrazolones

- Theoretical 56-65
- Discussion 66-75
- Experimental 76-78
- References 79-81

CHAPTER 4: Synthesis of steroidal benzothiazines and thiazoles

- Theoretical 82-88
- Discussion 89-98
- Experimental 99-101
- References 102-103

CHAPTER 5: Biological activities and DNA binding studies of newly synthesized heterosteroids

- Theoretical 104-108
- Antimicrobial activity 109-119
- Anticancer activity 120-144
- Antioxidant activity 145-148
- DNA binding studies 149-174
- References 175-179

Summary

THESIS

Steroids are a class of important polycyclic compounds which exhibit diverse biological activities. Except for the naturally occurring substances, most of steroidal pharmaceuticals are semi-synthetic compounds. It is proved that a number of biologically important properties of modified steroids are dependent upon structural features of the steroid ring system or side chain so this chemical modification of the steroid skeleton provides a way to alter the functional groups and numerous structure-activity relationships have been established by such synthetic alterations.

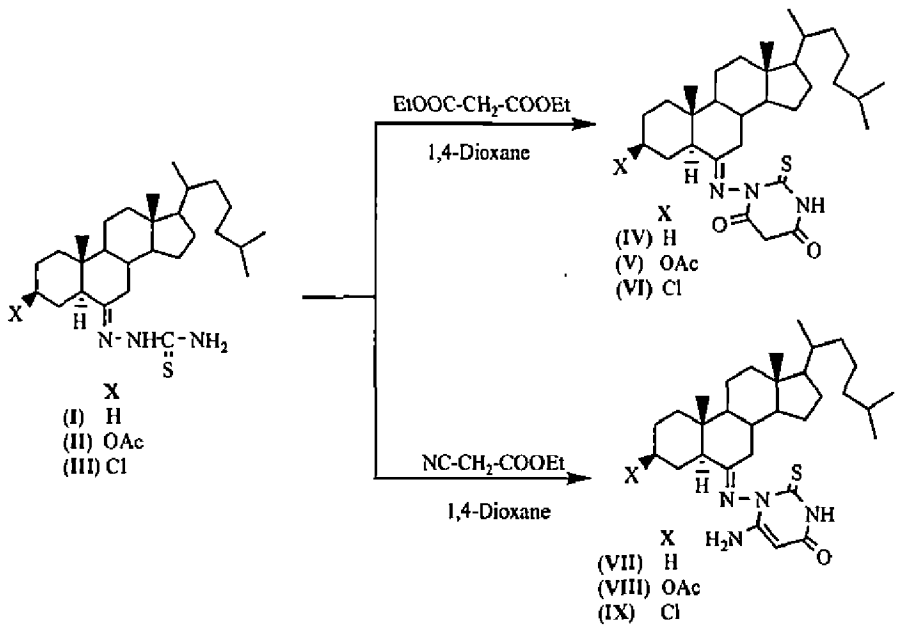
Steroids have always attracted considerable attention in bio-organic research because of being a fundamental class of biologically active molecules. Their profound physiological and clinical importance is now well validated. They can regulate a variety of biological processes and thus have the potential to act as drugs for the treatment of a number of diseases including cardiovascular, autoimmune, brain tumour, breast cancer, prostate cancer and osteoarthritis. The diversity in the biological action might be due to the presence of different functional groups located around the tetracyclic core which serve as substrates for different targets. The advantage of employing hydrophobic steroid units is their ability to interact with cell membranes and thus pave the way for biological activity of such hybrid molecules. Previous work from our laboratory described the synthesis of number of unknown steroidal derivatives in the cholestane series. These include azasteroids, oxasteroids, thiadiazoles, oxadiazolines, pyrrolidine, pyrazolines etc.

In continuation of the above work, an attempt has been made to synthesize new heterosteroids with probable biological activities like antimicrobial, anticancer, antioxidant and simultaneously study their DNA binding behavior. The newly synthesized heterosteroids have been characterized by IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. In some cases, abnormal products have been obtained and this has offered scope for some mechanistic and stereochemical studies also. The whole work of the thesis is divided into five chapters namely,

Chapter 1	:	Synthesis of steroidal pyrimidines
Chapter 2	:	Synthesis of steroidal pyrans
Chapter 3	:	Synthesis of steroidal pyrazoles and pyrazolones
Chapter 4	:	Synthesis of steroidal benzothiazines and thiazoles
Chapter 5	:	Biological activity (antimicrobial, anticancer and antioxidant) and DNA binding studies of newly synthesized heterosteroids

Synthesis of steroidal pyrimidines [1, 2]

Pyrimidine moiety containing molecules have been reported to exhibit a broad spectrum of biological activities such as anticancer, antiviral, antibacterial, antioxidant, anxiolytic, etc. As far as literature is concerned, little attention has been paid to the synthesis of steroidal based pyrimidine derivatives. This prompted us to carry out the synthesis of some steroidal pyrimidines and with this aim two series of reactions were carried out. First series involves the reactions of 5α-cholestan-6-one thiosemicarbazone (**I**), 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**II**) and 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**III**) with diethyl malonate to yield [4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**IV**), 3β-acetoxy [4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**V**) and 3β-chloro[4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**VI**), respectively while as the second series includes reactions of 5α-cholestan-6-one thiosemicarbazone and its analogues (**I-III**) with ethyl cyanoacetate to provide [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**VII**), 3β-acetoxy [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**VIII**) and 3β-chloro [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**IX**), respectively.

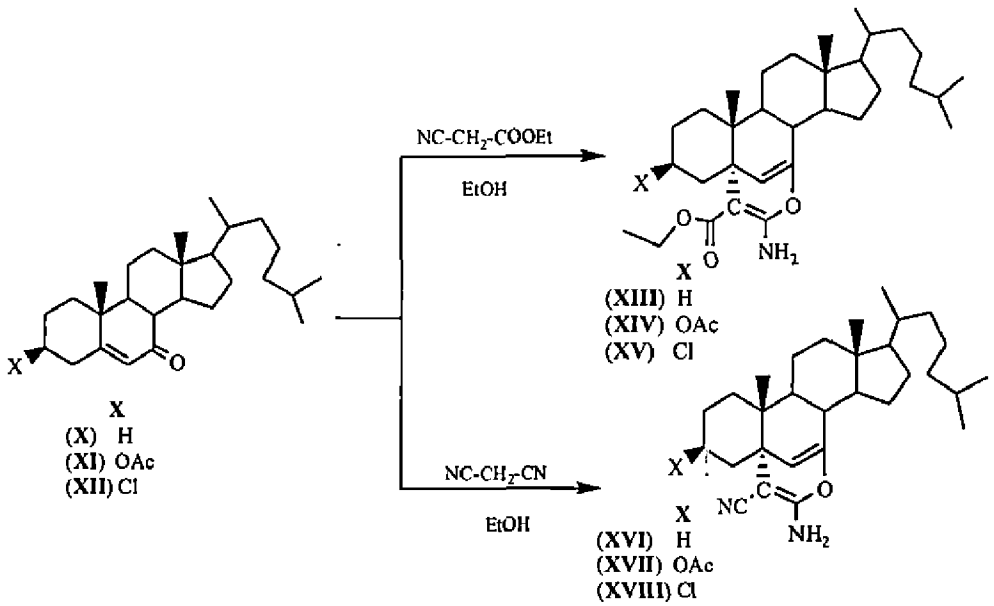


1. Shamsuzzaman, **Ayaz Mahmood Dar**, Z. Yaseen, K. Alam, A. Hussain, M. A. Gatoo, Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity. ***Journal of Molecular Structure*** 1045 (2013) 62-71
2. Shamsuzzaman, **Ayaz Mahmood Dar**, S. Tabassum, M. Zaki, Y. Khan, A. Sohail, M. A. Gatoo DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1H-pyrimidines. ***Comptes Rendus Chimie*** http://dx.doi.org/10.1016/j.crci.2013.07.001 (in press)

Chapter 2

Synthesis of steroidal pyrans [3, 4]

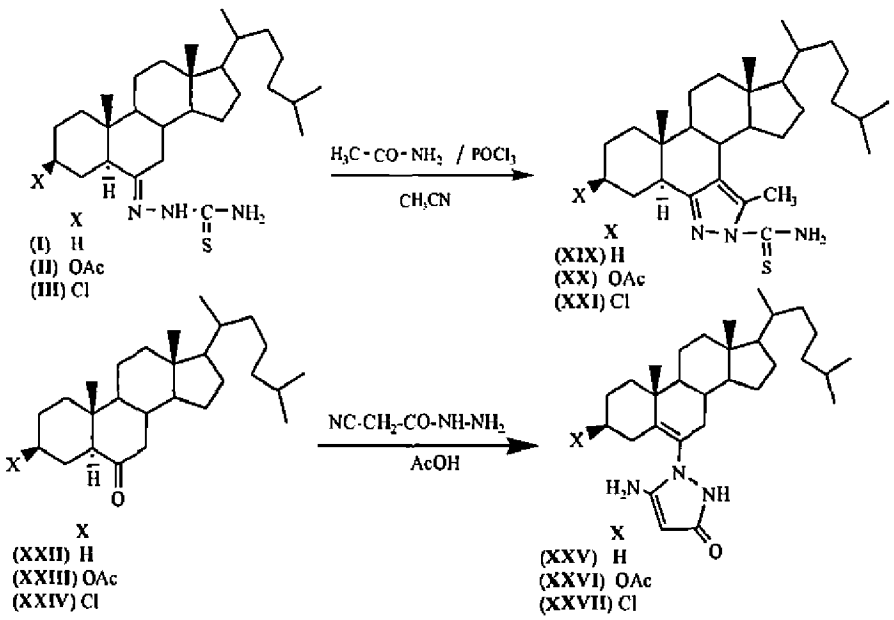
Pyran derivatives represent an important class of organic compounds with wide number of applications. They are not only used in cosmetics, pigments and biological agrochemicals but also constitute a structural unit of many natural products. These compounds have been reported to possess various pharmacological activities such as antiallergic, antitumor and antibacterial. The diversity in the biological action might be due to the presence of different functional moieties located around the pyran core which serve as substrates for different targets. Keeping this in consideration, the synthesis of some steroidal 4*H*-pyrans has been carried out and with this aim two series of reactions were done. First series involves the reactions of cholest-5-en-7-one (**X**), 3β-acetoxycholest-5-en-7-one (**XI**) and 3β-chlorocholest-5-en-7-one (**XII**) with ethyl cyanoacetate to yield 2'-amino-3'-carboethoxycholest-6-eno [5, 7- *d e*] 4H-pyran (**XIII**), 3β-acetoxy-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran (**XIV**) and 3β-chloro-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran (**XV**) while as the second series includes reactions of cholest-5-en-7-one and its analogues (**X-XII**) with malononitrile to provide 2'-amino-3'-cyanocholest-6-eno [5, 7 - *d e*] 4H-pyran (**XVI**), 3β-acetoxy-2'-amino-3'-cyanocholest-6-eno [5, 7 - *d e*] 4H-pyran (**XVII**) and 3β-chloro-2'-amino -3'-cyanocholest-6-eno [5, 7 - *d e*] 4H-pyran (**XVIII**), respectively.



3. Shamsuzzaman, **Ayaz Mahmood Dar**, A. Sohail, S. Bhat, M. F. Mustafa, Y. Khan, Synthesis, molecular docking and biological evaluation of new steroidal 4H-pyrans. ***Spectrochim. Acta Part A: Mol. Biomol. Spec.*** 117 (2014) 493-501
4. Shamsuzzaman, **Ayaz Mahmood Dar**, Y. Khan, A. Sohail, Synthesis and biological studies of steroidal pyran based derivatives. ***J. Photochem. Photobiol. Bio. B*** 129 (2013) 36-47

Synthesis of steroidal pyrazoles and pyrazolones [5, 6]

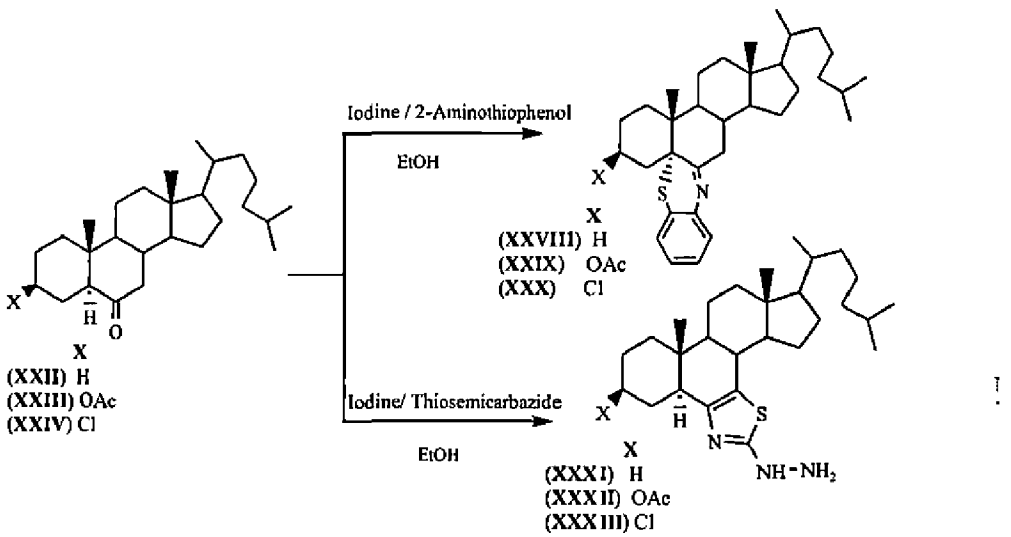
Pyrazoles have played a vital role in the development of theory in heterocyclic chemistry and are also used extensively as useful synthons in organic synthesis. Pyrazole derivatives also show number of biological activities like antimicrobial, anticancer, antioxidant, analgesic, anti-inflammatory, antipyretic, etc. This prompted us to carry out the synthesis of some of the steroidal pyrazoles and with this aim two series of reactions were done. First series involves the reactions of 5α-cholestan-6-one thiosemicarbazone (**I**), 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**II**) and 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**III**) with phosphorus oxychloride and acetamide to yield 5α-cholestano [6, 7- *c*] 5'-methyl-1'-carbo-thioic acid amido pyrazole (**XIX**), 3β-acetoxy-5α-cholestano [6, 7 - *c*] 5'-methyl-1'-carbo-thioic acid amido pyrazole (**XX**) and 3β-chloro-5α-cholestano [6, 7 - *c*] 5'-methyl-1'-carbo thioic acid amido pyrazole (**XXI**), respectively while as second series includes the reactions of 5α-cholestan-6-one (**XXII**), 3β-acetoxy-5α-cholestan-6-one (**XXIII**) and 3β-chloro-5α-cholestan-6-one (**XXIV**) with cyanoacetohydrazide to yield cholest-6 [5'-amino-1', 2'-dihydro pyrazol-3-one-1'-yl] 5-ene (**XXV**), 3β-acetoxycholest-6 [5'-amino-1', 2'-dihydropyrazol-3-one -1'-yl] 5-ene (**XXVI**) and 3β-chlorocholest-6 [5'-amino-1', 2'-dihydropyrazol-3-one-1'-yl] 5-ene (**XXVII**), respectively.



5. Shamsuzzaman, **Ayaz Mahmood Dar,** S. Shervani, I. Bhat, M. A. Gatoo, Structural, optical and antimicrobial studies of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones. ***J. Taibah University for Science*** http://dx.doi.org/10.1016/j.jtusci.2013.08.003, (in press) Elsevier
6. Shamsuzzaman, **Ayaz Mahmood Dar,** I. A. Bhat, Y. Khan, DNA binding studies and *in vitro* cytotoxicity of newly synthesized steroidal pyrazoles. (Communicated)

Synthesis of steroidal benzothiazines and thiazoles [7, 8]

Benzothiazines represent an important class of compounds in medicinal chemistry because the presence of nitrogen-sulphur axis is one of the features responsible for their biological activity; hence they show bioactivities like anticancer, vasorelaxant, antidiabetic, antihypertensive, antimicrobial, etc. On the other hand, thiazole derivatives have also attracted continuing interest over the years because of their varied biological activities. They have been reported as antiallergic, antihypertensive, anti-inflammatory, antischizophrenic, antibacterial, anti-HIV, hypnotics and selective COX-2 inhibitors, fibrinogen receptor antagonists with antithrombotic activity and new inhibitors of bacterial DNA gyrase B. With this enthusiasm, an attempt for the synthesis of these compounds was made and hence two series of reactions were carried out. First series involves the one pot synthesis of 5α-cholestano [5, 6 - *b*] benzothiazine (**XXVIII**), 3β-acetoxy-5α-cholestano [5, 6 - *b*] benzothiazine (**XXIX**) and 3β-chloro-5α-cholestano [5, 6 - *b*] benzothiazine (**XXX**) by reacting 5α-cholestan-6-one and its analogues (**XXII-XXIV**) with iodine/ 2-aminothiophenol while as the second series involves the one pot synthesis of 2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (**XXXI**), 3β-acetoxy-2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (**XXXII**) and 3β-chloro-2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (**XXXIII**) by reacting 5α-cholestan-6-one and its analogues (**XXII-XXIV**) with iodine/ thiosemicarbazide.



7. Shamsuzzaman, **Ayaz Mahmood Dar**, H. Khanam, M. A. Gatoo, Anticancer and antimicrobial evaluation of newly synthesized steroidal 5, 6 fused benzothiazines. ***Arabian Journal of Chemistry*** http://dx.doi.org/10.1016 /j.arabjc.2013.06.027, in press
8. Shamsuzzaman, **Ayaz Mahmood Dar**, H. Khanam, M. A. Gatoo, Synthesis, Characterization and *In Vitro* Anticancer Activity of Newly Synthesized Steroidal 6, 7-Fused Thiazoles. ***Journal of Chemistry*** (Accepted) (2013)

Chapter 5

Biological activities (antimicrobial, anticancer and antioxidant) and DNA binding studies of newly synthesized heterosteroids

Antimicrobial activity

In antimicrobial screening, the newly synthesized steroidal compounds showed moderate to potential behavior against different bacterial as well as fungal strains. During antimicrobial testing of steroidal pyrimidines (**IV-VI**), compound **IV** was found to be almost equally active as compared to the standard drug, Griseofulvin against *Candida albicans*. In case of screening of steroidal pyrans (**XIII-XV**) and steroidal pyrazolones (**XXV-XXVII**)[5] against different microbial strains, the activity of the compound **XIII** and **XXVI** was found to be almost same as the standard drugs, Ciprofloxacin and Chloramphenicol, respectively against the *Streptococcus pyogenes*. When steroidal benzothiazines (**XXVIII-XXX**)[7] were allowed to undergo the antimicrobial screening, all the three compounds showed moderate to good antibacterial activity but the compound **XXVIII** showed influential zone of inhibition *i.e.* 19.4 mm against the *E-coli* strain which is almost equal to the inhibition zone of Chloramphenicol *i.e.* 20.0 mm against the same strain.

Anticancer activity

During *in vitro* anticancer screening of steroidal pyrimidines (**IV-VI**), compound **IV** and **V** showed effective IC_{50} against A545, A549, HeLa and HepG2 cell lines hence showed potential cytotoxicity. In comet assay, compound **V** revealed maximum DNA damage against MCF7 cells. The nucleolytic experiment showed that reactive oxygen species ROS ($^{\cdot}OH$) are responsible for cell death.[1] In case of cytotoxicity assay of steroidal pyrimidines (**VII-IX**), compound **VIII** and **IX** showed IC_{50} against A549, HeLa, HepG2 and A545 cell lines close to that of Cisplatin, hence also show endowed cytotoxicity. Compound **VIII** allowed maximum DNA degradation in comet assay.[2]

During the cytotoxic screening of steroidal pyrans (**XIII-XV**), IC_{50} for **XIII** against HeLa and MCF7 was found to be 13.73 μmol L^{-1} and 11.18 μmol L^{-1} in comparison with Doxorubicin (IC_{50} = 11.53 μmol L^{-1} and 12.41 μmol L^{-1}), respectively. Hence these compounds also have cytotoxic potency and thus presented maximum DNA damage against MCF7 cells in the comet assay. The nucleolytic experiments showed that ROS ($^{\cdot}OH$) are responsible for cell death.[3] During the cytotoxic screening of steroidal pyrans (**XVI-XVIII**),

compound **XVII** was found to be potentially cytotoxic with its IC_{50} against MCF7 found to be 13.21 μmol L^{-1} (close to the IC_{50} of Doxorubicin 12.41 μmol L^{-1}) and hence presented maximum DNA degradation of MCF7 cells in comet assay. Microscopic examination of cancer cells and compound **XVII**-treated cancer cells also showed the inhibition of growth by compound **XVII**.[4]

In case of the *in vitro* cytotoxic screening of steroidal pyrazoles (**XIX-XXI**), compounds **XX** and **XXI** showed potential cytotoxicity against SW480, HL-60 and MCF-7 cell lines by showing IC_{50} close to that of Doxorubicin. Compound **XXI** showed IC_{50} same as that of Doxorubicin against HL-60 cell line. Microscopic examination of cancer cells and compound **XX** and **XXI**-treated cancer cells showed the cytotoxic nature of these compounds. In comet assay, compound **XX** presented higher DNA damage against MCF7 cells.[6]

During the cytotoxic screening of steroidal benzothiazines (**XXVIII-XXX**), compound **XXVIII** showed effective IC_{50} =13.73 μmol L^{-1} against HeLa cell line. Compounds **XXIX** and **XXX** also showed minimum IC_{50} of 15.83 μmol L^{-1} (HepG2) and 16.89 μmol L^{-1} (A549), respectively.[7] When the steroidal thiazoles (**XXXI-XXXIII**) were screened for the *in vitro* cytotoxicity, compound **XXXII** was found to be active against SW480, A549, HepG2, HeLa and HL-60 cells in comparison with the standard drug, Cisplatin.[8]

Antioxidant activity

2, 2-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay of new heterosteroids was studied during which the steroidal pyrimidines (**IV-VI**) exhibited potential antioxidant activity. DPPH assay of the steroidal pyrans (**XIII-XV**) was also carried out which showed that compounds were not significantly active. During the hydrogen peroxide (H_2O_2) scavenging assay, steroidal pyrazolones (**XVI-XVIII**) showed significantly active antioxidant behavior. When the nitric oxide radical scavenging assay of steroidal thiazoles (**XXXI-XXXIII**) was carried out, all the compounds showed potential nitric oxide radical scavenging behavior.

DNA binding studies

The DNA-binding studies included gel electrophoresis, UV-vis absorption and fluorescence spectroscopy and molecular docking technique. The binding constants (K_b) for the steroidal pyrimidines (**IV-IX**) were found to be 4.63×10^{-3} M^{-1}, 2.12×10^{-3} M^{-1}, 2.43×10^{-3} M^{-1}, 9.34×10^{3} M^{-1}, 6.56×10^{3} M^{-1} and 1.54×10^{4} M^{-1}, respectively. It was found that the absorption intensity in the compounds (**IV-IX**) increased with gradual addition of DNA and hence

showed hyperchromism, revealing the fact of exposure of more bases of DNA which is the indication of strong binding of compounds to CT DNA.[1,2]

The binding constants (K_b) for the steroidal pyrans **XIV** and **XV** were found to be 5.3×10^3 and 3.7×10^3 M^{-1} and for the steroidal pyrans (**XVI-XVIII**) K_b was found to be 2.2×10^3 M^{-1}, 5.37×10^3 M^{-1} and 2.51×10^3 M^{-1}, respectively. The absorption spectra of these compounds also revealed strong hyperchromism implying their higher DNA binding propensity which results in partial uncoiling of DNA helix structure, exposing more bases of DNA which is the indication of strong binding of compounds to CT DNA.[3,4]

The gel electrophoresis also revealed the interaction of these compounds with DNA by showing the disappearance of the original band of DNA in gel plate. In molecular docking study, it was found that these type of compounds interacted with DNA through major as well as minor groove and their hetereocyclic moiety (pyrimidine or pyran) shows electrostatic interaction between the nucleotide base pairs of DNA.[1-4]

List of Publications

1. Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity, Shamsuzzaman, **Ayaz Mahmood Dar**, Zahid Yaseen, Khursheed Alam, Altaf Hussain, Manzoor Ahmad Gatoo. *Journal of Molecular Structure* 1045 (2013) 62-71

2. DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1H-pyrimidines, Shamsuzzaman, **Ayaz Mahmood Dar**, Sartaj Tabassum, Mehvash Zaki, Yusuf Khan, Aamir Sohail, Manzoor Ahmad Gatoo. *Comptes Rendus Chimie*, http://dx.doi.org/10.1016/j.crci.2013.07.001 (in press)

3. Synthesis, molecular docking and biological evaluation of new steroidal 4H-pyrans, Shamsuzzaman, **Ayaz Mahmood Dar**, Aamir Sohail, Sheraz Bhat, Mir Faisal Mustafa, Yusuf Khan, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 117 (2014) 493-501

4. Synthesis and biological studies of steroidal pyran based derivatives. Shamsuzzaman, **Ayaz Mahmood Dar**, Yusuf Khan, Aamir Sohail, *Journal of Photochemistry and Photobiology B* 129 (2013) 36-47

5. Anticancer and antimicrobial evaluation of newly synthesized steroidal 5, 6 fused benzothiazines. Shamsuzzaman, **Ayaz Mahmood Dar**, Hena Khanam, Manzoor Ahmad Gatoo, *Arabian Journal of Chemistry* http://dx.doi.org/10.1016/j.arabjc.2013.06.027 (in press)

6. Structural, optical and antimicrobial studies of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones. Shamsuzzaman, **Ayaz Mahmood Dar**, Suboohi Shervani, Irshad Bhat, Manzoor Ahmad Gatoo, *Journal of Taibah University for Science*, http://dx.doi.org/10.1016/j.jtusci.2013.08.003 (in press)

7. Synthesis, evaluation and docking studies on steroidal pyrazolones as anticancer and antimicrobial agents. Shamsuzzaman, Ashraf Mashrai, Anis Ahmad, **Ayaz Mahmood Dar**, Hena Khanam, Mohd Danishuddin, Asad U. Khan, *Medicinal Chemistry Research*, DOI 10.1007/s00044-013-0636-y (in press)

8. Synthesis, characterization, antimicrobial and anticancer studies of new steroidal pyrazolines. Shamsuzzaman, Hena Khanam, **Ayaz Mahmood Dar**, Nazish Siddiqui, Sumbul Rehman, *Journal of Saudi Chemical Society*, http://dx.doi.org/10.1016/j.jscs.2012.05.004 (in press)

9. Synthesis, characterization and anticancer studies of new steroidal oxadiazole, pyrrole and pyrazole derivatives. Shamsuzzaman, Tabassum Siddiqui, Mohd Gulfam Alam, **Ayaz Mahmood Dar**, *Journal of Saudi Chemical Society* http://dx.doi.org/10.1016/j.jscs.2012.04.009 (in press)

10. Synthesis, characterization and in vitro anticancer activity of newly synthesized steroidal 6, 7-fused thiazoles. Shamsuzzaman, **Ayaz Mahmood Dar**, Hena Khanam, Manzoor Ahmad Gatoo, *Journal of Chemistry* (2013) accepted

Introduction

Steroids are a group of chemical substances which are found abundant in nature and show wide range of characteristics. They include cholesterol, sex hormones, adrenal cortex hormones, bile salts, oestrogens, vitamin-D, sapogenin and some other important metabolites. These steroids have a close association or rather indispensable for various physiological phenomena of the living world thus making themselves the focus of attention in the field of organic chemistry. The incentive feature of steroids is that they can be easily synthesized in the laboratory on a large scale. The wide implication of these compounds and their association with life has made the field of steroids as an important research area. The discovery of testosterone which showed marked biological properties is the most striking feature of this field. Scientists diverted their attention towards the steroids and the work on isolation as well as on synthesis started with great enthusiasm. Thereafter, researchers diversified their efforts to evaluate the structural and stereochemical problems of steroidal skeleton because the stereochemistry is the primary factor responsible for the physiological activity of the steroids. Later on the structure elucidation and the reaction mechanism of the steroids became a matter of immense importance.

The fusion of any heterocyclic ring system to steroid core or to introduce any hetero atom such as sulfur, oxygen, nitrogen or any of the halogen group members, was found to augment the biological and industrial revolution. Hence, innumerable methods started developing across the world to find the better substitutes for already existing steroids. As a matter of fact, steroid chemistry has always proved to be much inviting to chemists and industries and also fascinated us to undertake the work in the field. Our laboratory is mainly concerned with the synthesis of steroidal compounds and their identification by chemical and spectral studies, has reported the preparation of a number of heterosteroids.

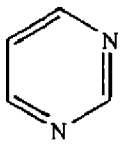
In the present work an attempt has been made to synthesize some modified steroids of biological interest like pyrimidines, pyrans, pyrazoles, pyrazolones, benzothiazines and thiazoles. First chapter is dealing with the synthesis of steroidal pyrimidines, second chapter is concerned with synthesis of steroidal pyrans, third chapter is attributed to the synthesis of steroidal pyrazoles and pyrazolones while as the fourth chapter is associated with the synthesis of steroidal benzothiazines and thiazoles. The fifth chapter is concerned with the biological activities (antimicrobial, anticancer and antioxidant) and DNA binding studies of these newly synthesized heterosteroid compounds.

Chapter-1

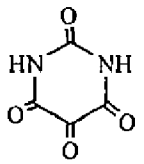
Synthesis of steroidal pyrimidines

Theoretical

Pyrimidine or 1, 3-diazine (1) is a 6-membered aromatic heterocycle with two nitrogen atoms in the ring at 1, 3-positions.[1] It may be regarded as being derived from benzene by the replacement of two *meta* "CH" groups by "N". The pyrimidine ring system has wide occurrence in nature as substituted and fused ring derivatives, like the nucleotides, thiamine and alloxan (2).[2] It is also found in many synthetic compounds such as barbiturates and the HIV drug, zidovudine.

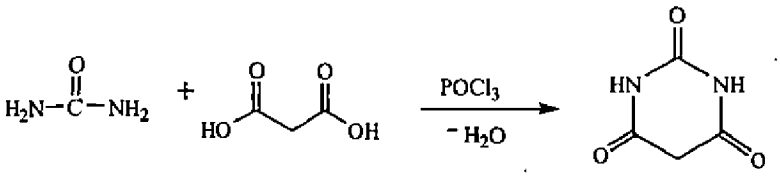


(1)



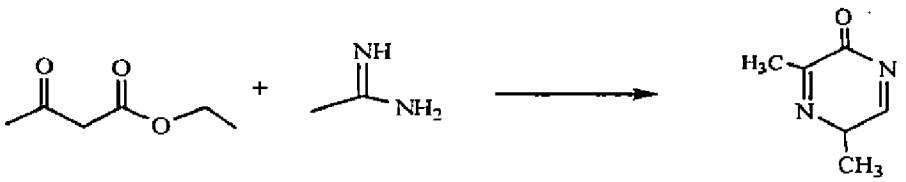
(2)

Although pyrimidine derivatives such as uric acid and alloxan were known in the early 19th century, a laboratory synthesis of a pyrimidine was not carried out until 1879,[2] when Grimaux[3] reported the preparation of barbituric acid (3) from urea and malonic acid in the presence of phosphorus oxychloride.



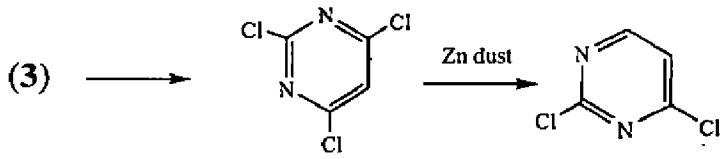
(3)

The systematic study of pyrimidines began in 1884 with Pinner[4] who synthesized 3, 5-dimethyl-5H-pyrazin-2-one (4) by condensing ethyl acetoacetate with acetamidine. Pinner[5] first proposed the name "pyrimidin" in 1885 which later got modified as "pyrimidine".



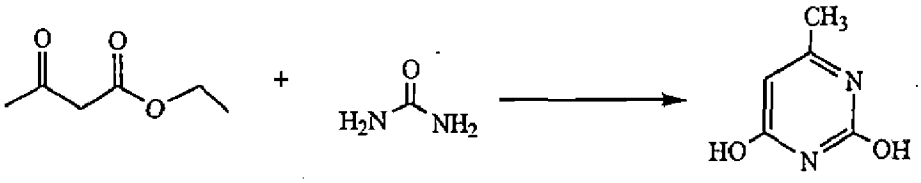
(4)

Gabriel and Colman[6] in 1900 reported the conversion of barbituric acid (3) into trichloropyrimidine (5) by $POCl_3$. The compound (5) upon selective reduction yielded dichloropyrimidine (6).



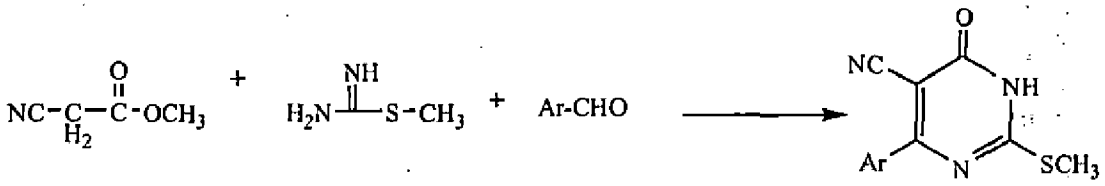
(5) (6)

Benhard[7] reported the reaction of ethyl acetoacetate with urea that yielded a pyrimidine derivative (4-methyluracil) (**7**).



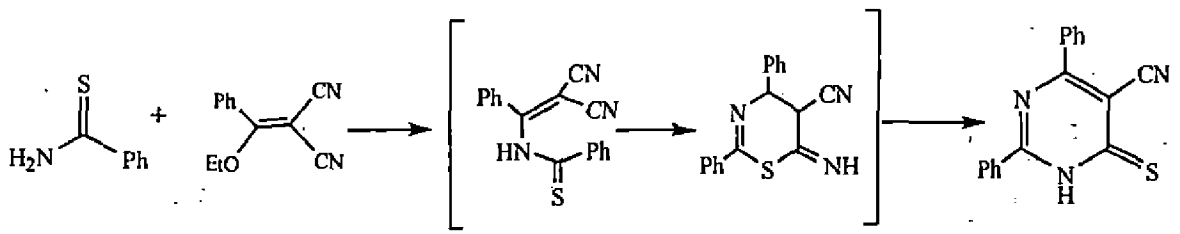
(7)

Hussain and co-workers[8] reported that heating the mixture of methyl cyanoacetate, S-methyl isothiourea and aldehydes (**8 a-d**) yielded corresponding 4-aryl-5-cyano-2-methylthio-6-oxopyrimidine derivatives (**9 a-d**).



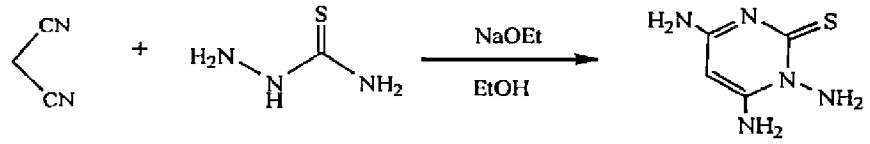
(8) Ar	(9) Ar
(a) C_6H_5	(a) C_6H_5
(b) p-NO_2-C_6H_4	(b) p-NO_2-C_6H_4
(c) o-CH_3O-C_6H_4	(c) o-CH_3O-C_6H_4
(d) o-NO_2-C_6H_4	(d) o-NO_2-C_6H_4

Soto and co-workers[9] reported the reaction of thiobenzamide with 3-ethoxy-3-phenyl-2-cyanoacrylonitrile (**10**) in presence of sodium isopropoxide in 2-propanol to afford 5-cyano-2, 6-diphenyl-4-thioxo-3, 4-dihydropyrimidine (**11**) through formation of the 3-phenyl-2-cyano-3-thiobenzamide acrylonitrile as an intermediate.



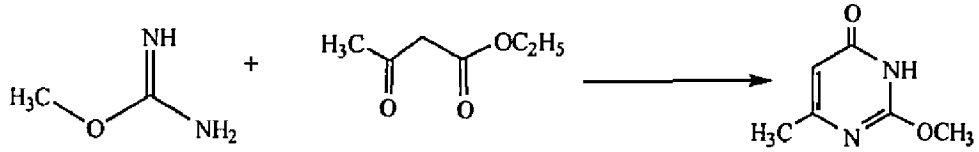
(10) (11)

Taylor and Morrison[10] reported the synthesis of 1, 4, 6-triamino-2(2H)-pyrimidine-2-thione (**12**) by reacting malononitrile with thiosemicarbazide in presence of sodium ethoxide in ethanol.



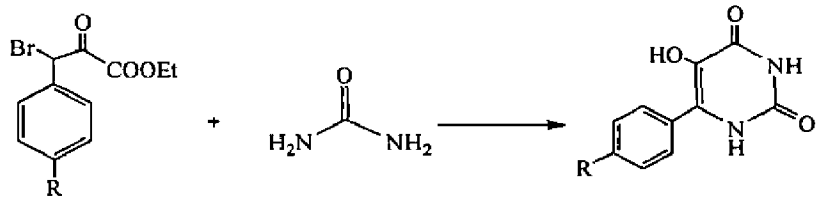
(12)

Botta and co-workers[11] reported the synthesis of 2-methoxy-6-methyl-3(2H)-pyrimidin-4-one (**13**) after reacting ethyl acetoacetate and O-methylisourea in an aqueous medium.



(13)

Andereichikov and co-workers[12] reported the synthesis of uracil derivatives (**15 a-d**) by the reaction of aryl substituted bromopyruvate esters (**14 a-d**) with urea.



(14)

R

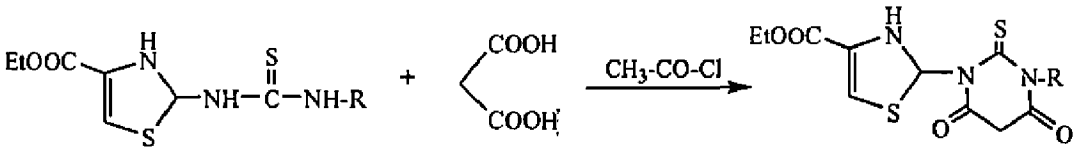
(a) OCH_3
(b) OH
(c) Cl
(d) H

(15)

R

(a) OCH_3
(b) OH
(c) Cl
(d) H

El-Subbagh[13] reported the synthesis of pyrimidine derivative (**17 a, b**) by reacting thiazolyl thiourea derivatives (**16 a, b**) with malonic acid in the presence of acetyl chloride.



(16)

R

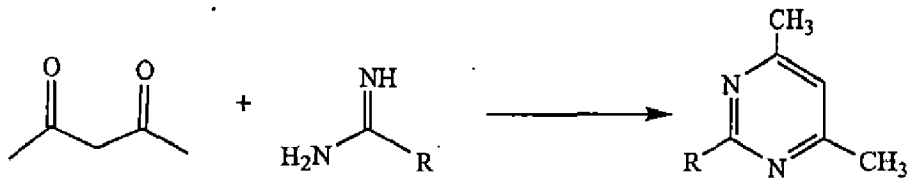
(a) CH_3
(b) C_2H_5

(17)

R

(a) CH_3
(b) C_2H_5

Bowman and co-workers[14] reported that acetyl acetone condensed with acetamidine derivatives (**18 a-e**) and gave corresponding pyrimidine derivatives (**19 a-e**) in good yields.



(18)

R

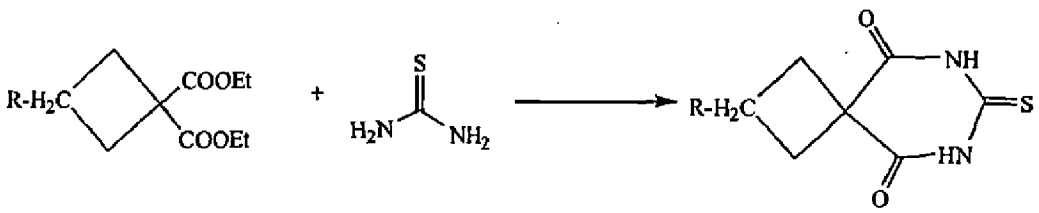
(a) $NH\text{-}C_6H_5$
(b) SH
(c) $NH\text{-}C_6H_4CH_3$
(d) $NHNH.NO_2$
(e) C_2H_5

(19)

R

(a) $NH\text{-}C_6H_5$
(b) SH
(c) $NH\text{-}C_6H_4CH_3$
(d) $NHNH.NO_2$
(e) C_2H_5

Yossef and co-workers[15] reported the reaction of 1, 1-cycloalkane dicarboxylic acid diethyl esters (**20 a-c**) with thiourea which gave *spiro*-barbituric acid derivatives (**21 a-c**).



(20)

R

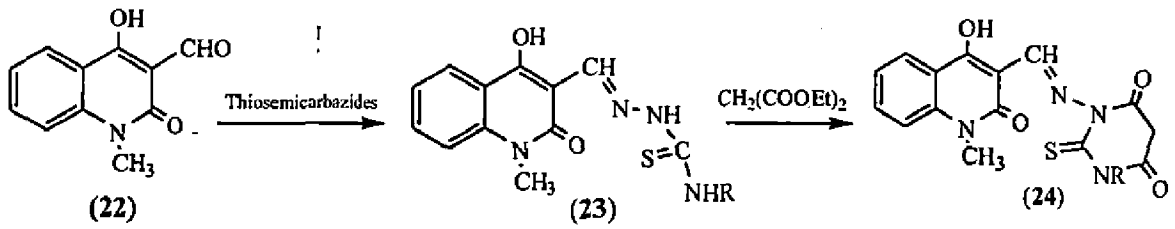
(a) CH_3
(b) C_2H_5
(c) Isopropyl

(21)

R

(a) CH_3
(b) C_2H_5
(c) Isopropyl

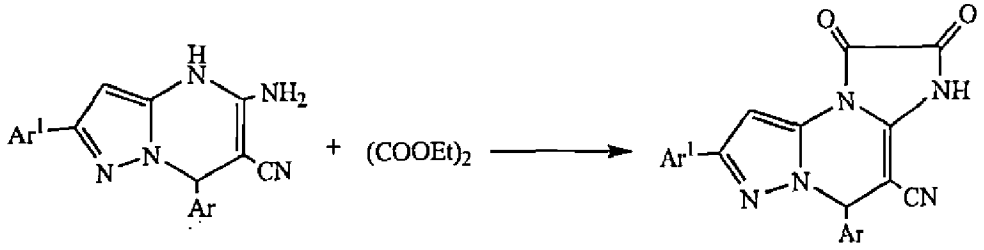
Mohamed[16] reported the reaction of 1, 2-dihydro-4-hydroxy-1-methyl-2-oxoquinoline-3-carbaldehyde (**22**) with thiosemicarbazides to yield desired thiosemicarbazones (**23 a-d**). These thiosemicarbazones then reacted with diethyl malonate which resulted into corresponding pyrimidine derivatives (**24 a-d**) in good yields.



(22) (23) (24)

R= (a) H (b) C_6H_5 (c) *p*-$CH_3O\text{-}C_6H_4$ (d) $H_2C{=}CH\text{-}CH_2$

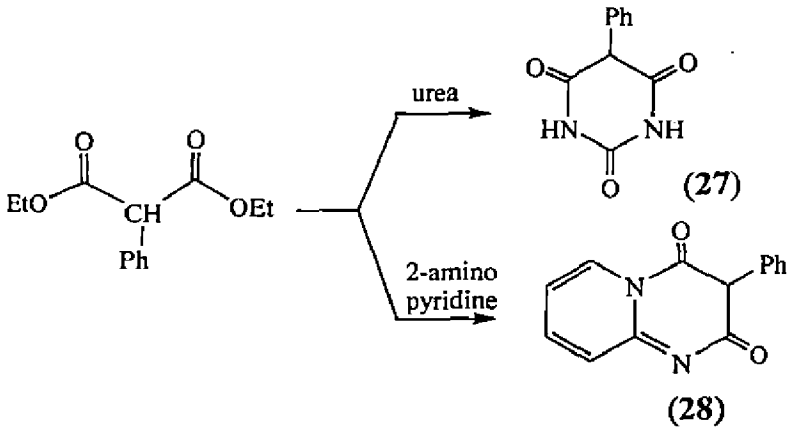
Eldin and Attaby[17] reported the reaction of pyrazole [3, 2 - *b*] pyrimidine derivatives (**25 a-p**) with diethyl oxalate which yielded imidazo [1, 2:3′, 4′] pyrazole [3, 2 - *b*] pyrimidine derivatives (**26 a-p**) in good yields.



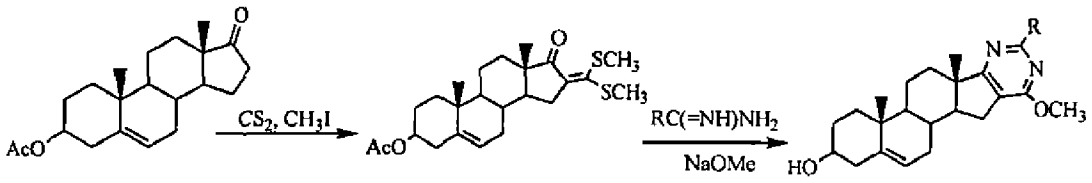
(25) (26)

Ar	Ar[1]	Ar	Ar[1]
(a) C_6H_5	C_6H_5	(a) C_6H_5	C_6H_5
(b) *p*-Cl-C_6H_4	C_6H_5	(b) *p*-Cl-C_6H_4	C_6H_5
(c) *p*-OCH_3-C_6H_4	C_6H_5	(c) *p*-OCH_3-C_6H_4	C_6H_5
(d) *p*-NO_2-C_6H_4	C_6H_5	(d) *p*-NO_2-C_6H_4	C_6H_5
(e) C_4H_3O-*α*	C_6H_5	(e) C_4H_3O-*α*	C_6H
(f) C_4H_3S-*α*	C_6H_5	(f) C_4H_3S-*α*	C_6H_5
(g) C_6H_5	*p*-CH_3-C_6H_4	(g) C_6H_5	*p*-CH_3-C_6H_4
(h) *p*-Cl-C_6H_4	*p*-CH_3-C_6H_4	(h) *p*-Cl-C_6H_4	*p*-CH_3-C_6H_4
(i) *p*-OCH_3-C_6H_4	*p*-CH_3-C_6H_4	(i) *p*-OCH_3-C_6H_4	*p*-CH_3-C_6H_4
(j) *p*-NO_2-C_6H_4	*p*-CH_3-C_6H_4	(j) *p*-NO_2-C_6H_4	*p*-CH_3-C_6H_4
(k) C_4H_3O-*α*	*p*-CH_3-C_6H_4	(k) C_4H_3O-*α*	*p*-CH_3-C_6H_4
(l) C_4H_3S-*α*	*p*-CH_3-C_6H_4	(l) C_4H_3S-*α*	*p*-CH_3-C_6H_4
(m) C_6H_5	*p*-Br-C_6H_4	(m) C_6H_5	*p*-Br-C_6H_4
(n) *p*-Cl-C_6H_4	*p*-Br-C_6H_4	(n) *p*-Cl-C_6H_4	*p*-Br-C_6H_4
(o) *p*-OCH_3-C_6H_4	*p*-Br-C_6H_4	(o) *p*-OCH_3-C_6H_4	*p*-Br-C_6H_4
(p) C_4H_3O-*α*	*p*-Br-C_6H_4	(p) C_4H_3O-*α*	*p*-Br-C_6H_4

Stadlbauer and co-workers[18] reported the reaction of diethyl malonate with urea and 2-aminopyridine which gave barbituric acid derivative (**27**) and pyrido [1, 2 - *a*] pyrimidine-2, 4-dione derivative (**28**), respectively in better yields.



Peseke and co-workers[19] reported the reaction of androstenolone acetate (29) with CS_2 and CH_3I that yielded 3β-acetoxy-16-[bis(methylthio) methylene] androst-5-en-17-one (30) which later reacted with different amidines in presence of sodium methoxide to provide corresponding steroidal pyrimidine derivatives (31 a-e).

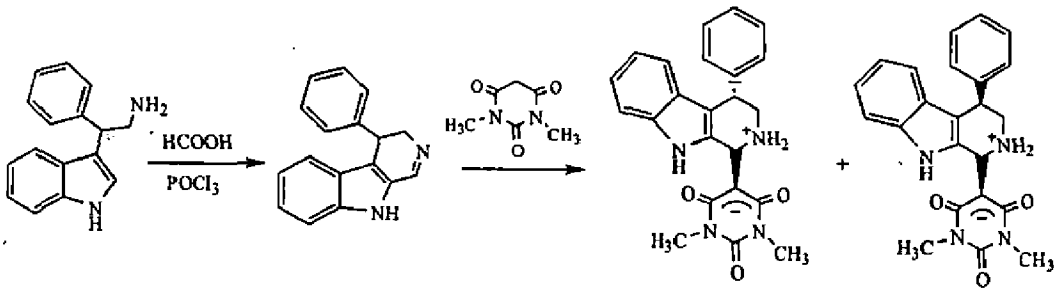


(29) (30) (31)

R

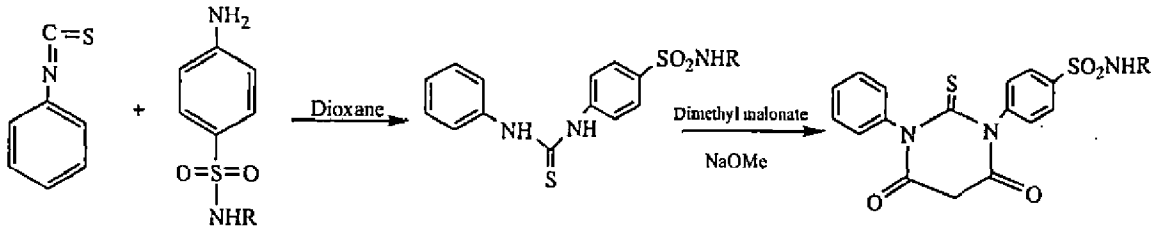
(a) H
(b) C_2H_5
(c) C_6H_5
(d) NH_2
(e) OCH_3

Semenov *et al.*,[20] reported the reaction of β-phenyl tryptamine (32) with formic acid under Bischler-Napieralski conditions that yielded 4-phenyl-3, 4-dihydro-β-carboline (33) which upon reaction with 1, 3-dimethylbarbituric acid provided two diastereomers of substituted pyrimidinones [34 (*RR*), 35 (*RS*)].



(32) (33) (34) (35)

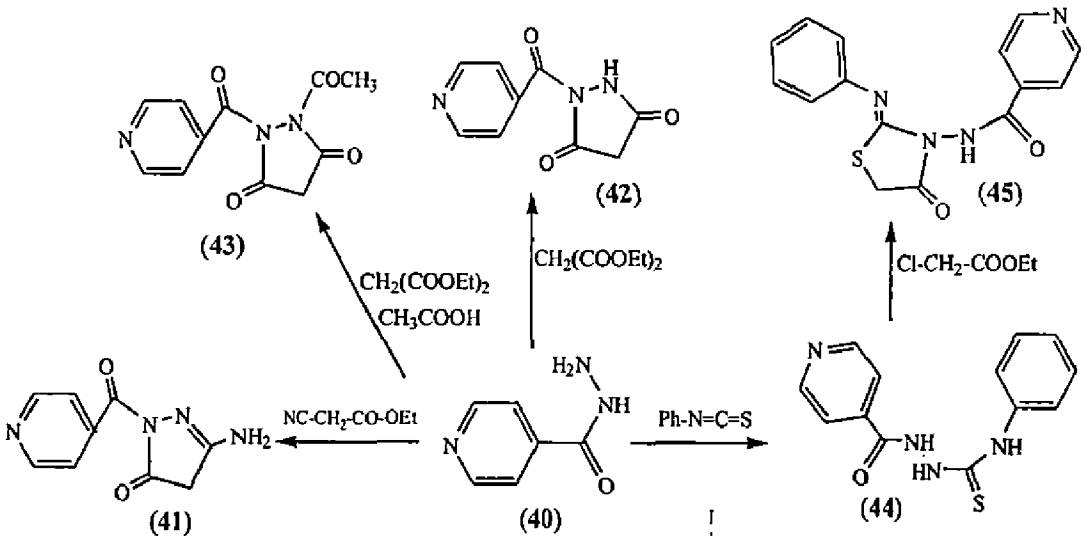
Bawazir and co-workers[21] reported the reaction of phenylisothiocyanate (36) with sulfa drugs (37 a-c) in 1, 4-dioxane that yielded N, N'-disubstituted thiourea derivatives (38 a-c) which later on reaction with dimethyl malonate in presence of sodium methoxide provided corresponding 1, 3-disubstituted thiobarbituric acid derivatives (39 a-c).



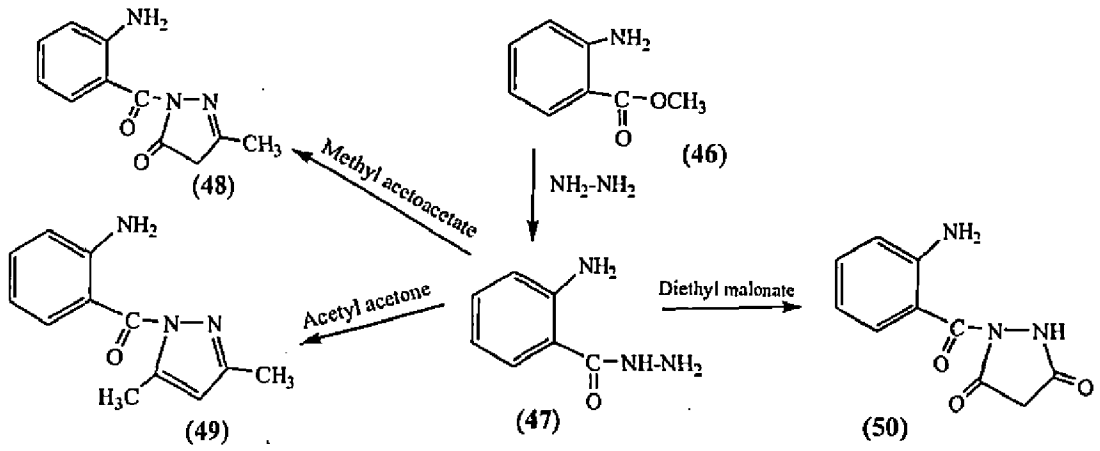
(36) (37) (38) (39)

	R	R	R
(a)	H	H	H
(b)			
(c)			

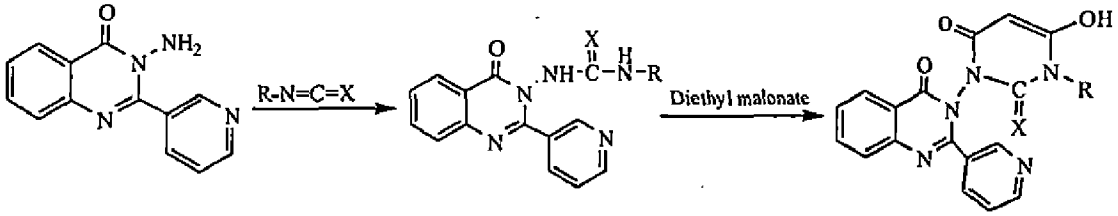
Parashar and co-workers[22] reported the reaction of isonicotinohydrazide (40) with ethyl cyanoacetate, diethyl malonate, diethyl malonate with acetic acid and phenylisothiocyanate, which gave 3-amino-1-isonicotinoyl-1H-pyrazol-5(4H)-one (41), 1-isonicotinoyl pyrazolidine-3, 5-dione (42), 1-acetyl-2-isonicotinoylpyrazolidine-3, 5-dione (43), 1-isonicotinoyl-4-phenylthiosemicarbazide (44), respectively. The compound (44) on further reaction with ethyl chloroacetate gave N-(4-oxo-2-(phenylimino) thiazolidin-3-yl) isonicotinamide (45).



Hassan[23] reported the reaction of methyl anthranilate (46) with hydrazine hydrate that gave 2-aminobenzhydrazide (47) which in turn on reaction with methyl acetoacetate, acetyl acetone and diethyl malonate, yielded (2-amino benzoyl)-3-methyl-1H-pyrazole-5-(4H)-one (48), (2-aminobenzoyl)(3, 5-dimethyl-1H-pyrazole-1-yl) methanone (49) and (2-amino benzoyl) pyrazolidine-3, 5-dione (50), respectively in good amounts.



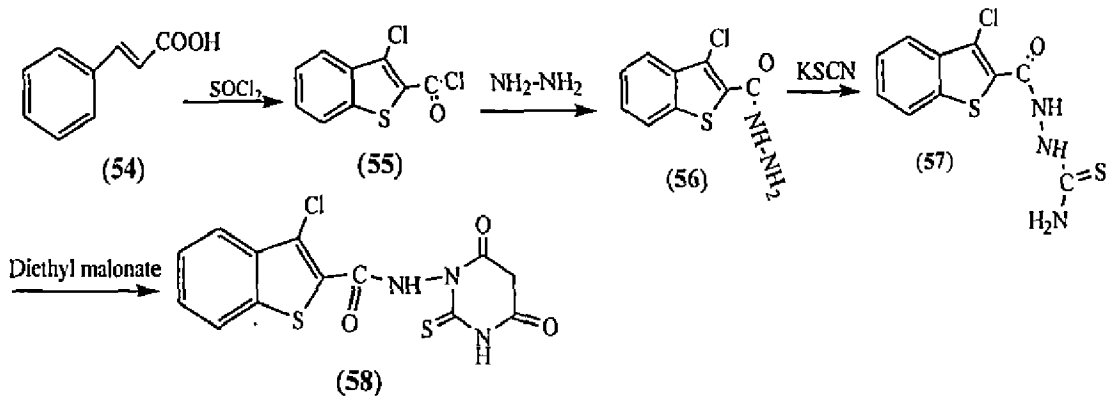
Abbas and co-workers[24] reported the reaction of 3-amino-2-(pyridin-3-yl)-4-quinazolinone (**51**) with *p*-hydroxy phenylisocyanate and phenylisothiocyanate provided 1-(4-oxo-2-(pyridin-3-yl)-quinazolin-3(4H)-yl)-3-phenylurea (**52a**) and 1-(4-oxo-2-(pyridin-3-yl)-quinazolin-3(4H)-yl)-3-phenylthiourea (**52b**). The compounds (**52a** and **52b**) upon reaction with diethyl malonate and after keto-enol tautomerism, yielded 6-hydroxy-3-(4-oxo-2-(pyridin-3-yl)-quinazolin-3-(4H)-yl)-1-phenyl pyrimidine-2, 4-(1H, 3H)-dione (**53a**) and 3-(4-hydroxy-6-oxo-3-phenyl-2-thioxo-2, 3-dihydropyrimidin-1(6H)-yl)-2-(pyridin-3-yl)-quinazolin-4(3H)-one (**53b**).



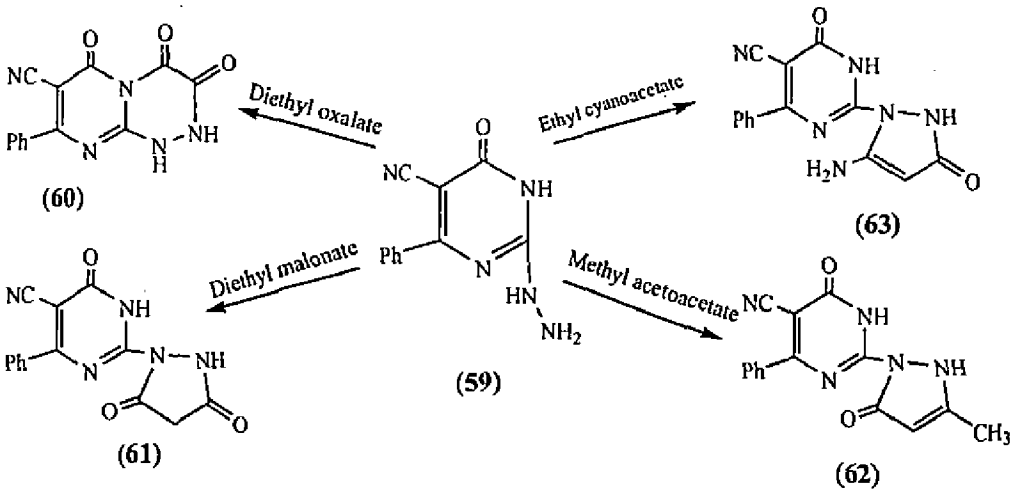
(51) (52) (53)

	(52) X	(52) R	(53) X	(53) R
(a)	O	C_6H_5	O	C_6H_5
(b)	S	C_6H_4-*p*OH	S	C_6H_4-*p*OH

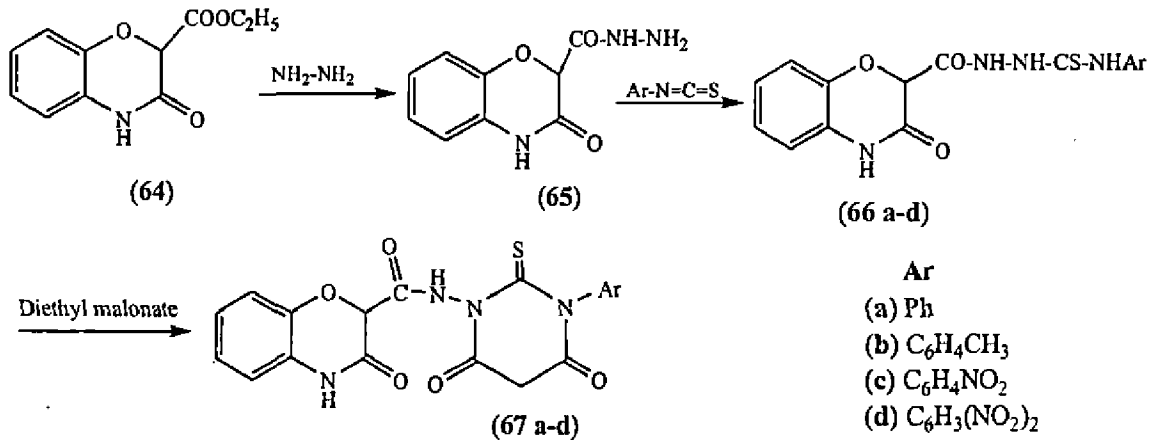
Naganagowda and co-workers[25] reported the synthesis of 3-chloro-1-benzothiophene-2-carbonylchloride (**55**) from cinnamic acid (**54**). The compound (**55**) reacted with hydrazine and gave 3-chloro-1-benzo [*b*] thiophene-2-carboxylic acid hydrazide (**56**) which on reaction with potassium thiocyanate provided 2-[(3-chloro-1-benzo [*b*] thiophen-2-yl) carbonyl] hydrazine carbothioamide (**57**) which on reaction with diethyl malonate yielded 3-chloro-N(4, 6-dioxo-2-thioxotetrahydropyrimidin-1(2H)-yl)-1-benzothiophene-2-carboxamide (**58**).



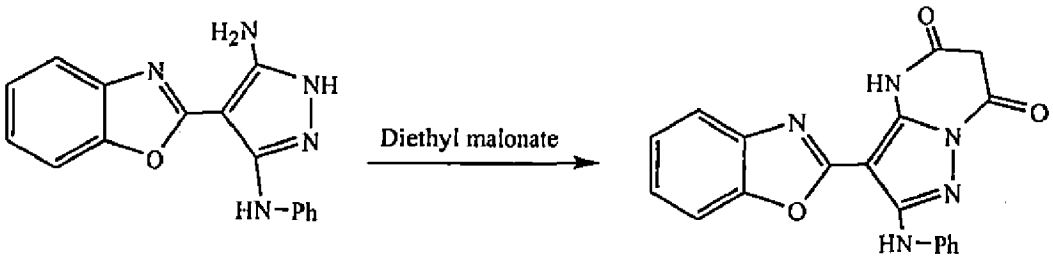
El-zahar and co-workers[26] reported the reaction of 2-hydrazino-6-oxo-4-phenyl-1, 6-dihydropyrimidine-5-carbonitrile (**59**) with diethyl oxalate, diethyl malonate, methyl acetoacetate and ethyl cyanoacetate that provided corresponding 3, 4, 6-trioxo-8-phenyl-tetrahydro-2H-pyrimido[2, 1 - *c*][1, 2, 4] triazine-7-carbonitrile (**60**), 2-(3, 5-dioxo-pyrazo lidin-1-yl)-6-oxo-4-phenyl-1, 6-dihydropyrimidine-5-carbonitrile (**61**), 2-(3-methyl-5-oxo-2, 5-dihydro-pyrazol-1-yl)-6-oxo-4-phenyl-1, 6-dihydropyrimidine carbonitrile (**62**) and 2-(5-amino-3-oxo-2, 3-dihydro-pyrazol-1-yl)-6-oxo-4-phenyl-1, 6-dihydropyrimidine-5-carbonitri -ile (**63**), respectively in good yields.



Dabholkar and Gavande[27] reported the reaction of 2H, 4H-2-ethoxycarbonyl-3, 4-dihydro-3-oxo-1, 4-benzoxazine (**64**) with hydrazine and provided 2H, 4H-2-hydrazino carbonyl-3, 4-dihydro-3-oxo-1, 4-benzoxazine (**65**) which upon reaction with arylisothiocyanate derivatives yielded 2H, 4H-2-[(4′-substituted)-arylthiosemicarbazino]-carbonyl-3, 4-dihydro-3-oxo-1, 4-benzoxazine derivatives (**66 a-d**). The compounds (**66 a-d**) later on reaction with diethyl malonate and gave 2H, 4H-2-[5′H-5′-dihydro-2′-thioxo-3′-aryl-4′, 6′-dioxo-1, 3-diazine]-aminocarbonyl-3, 4-dihydro-3-oxo-1, 4-benzoxazines (**67 a-d**).

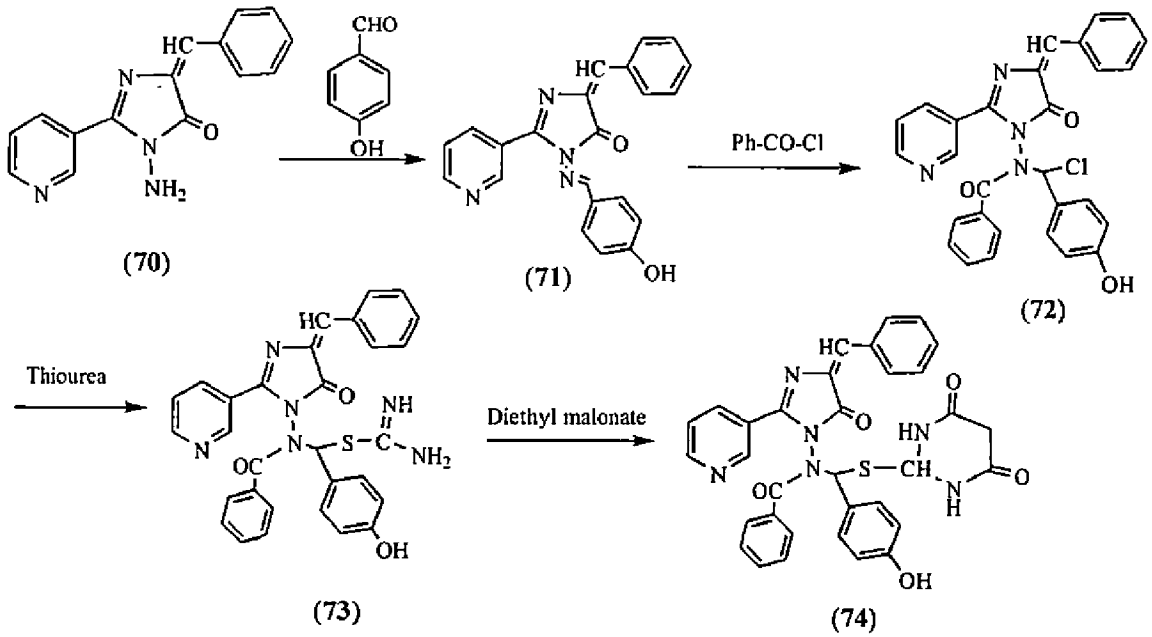


Wahab and Mohamed[28] reported the reaction of N-[3-amino-4-(benzoxazol-2-yl) pyrazol-5-yl] phenylamine (**68**) with diethyl malonate which provided 3-benzoxazol-2-yl-2-phenylamino-4H-pyrazolo [1, 5 - *a*] pyrimidine-5, 7-dione (**69**) in 80% yield.

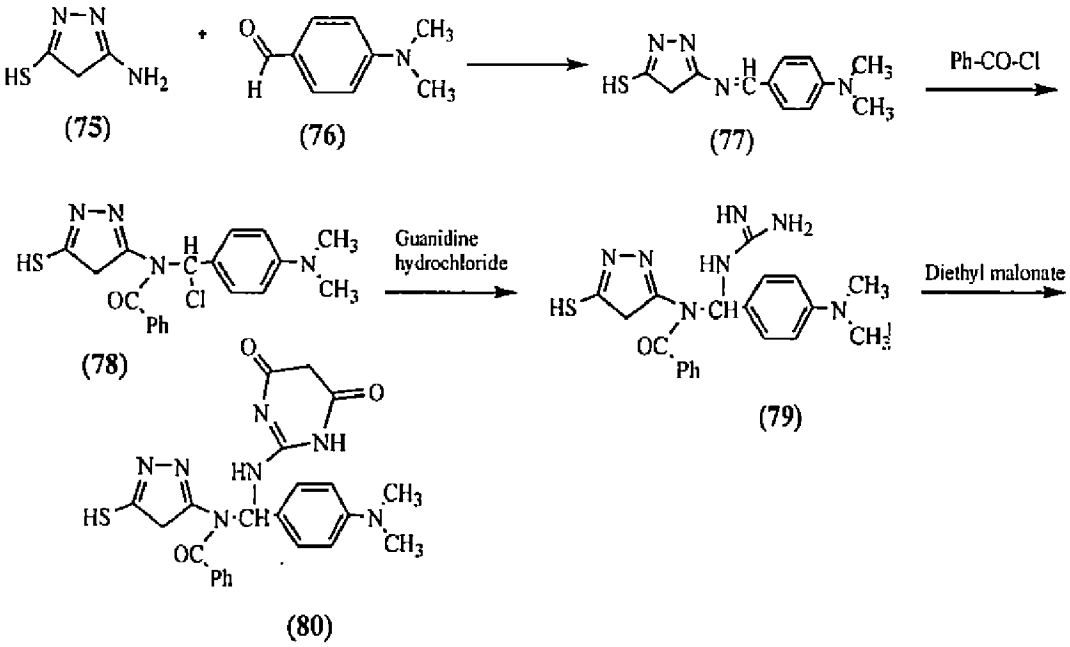


(**68**) (**69**)

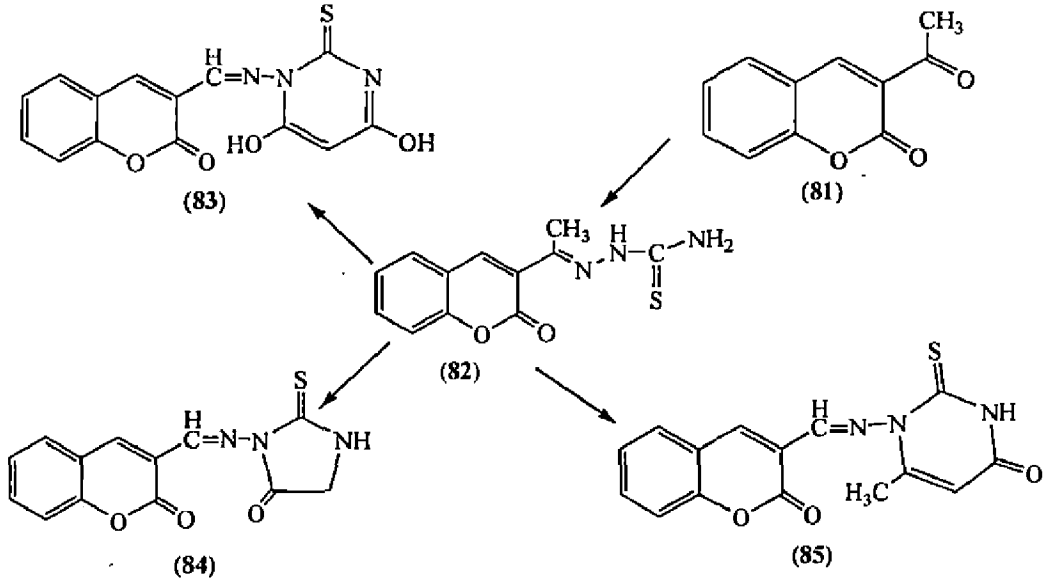
Abodi and co-workers[29] reported the reaction of 3-amino-5-(benzylidene)-2-(pyridin-3-yl)-3, 5-dihydro-4H-imidazol-4-one (**70**) with *p*-hydroxybenzaldehyde which gave 5-(benzylidene)-3-[(*p*-hydroxybenzylidene) amino]-2-(pyridin-3-yl) dihydro-4H-imidazol-4-one (**71**). The compound (**71**) reacted with benzoyl chloride and provided N-[chloro (4′-phenyl) methyl]-N-[4′-*p*-hydroxybenzylidene-5′-oxo-2-(pyridin-3-yl)-4, 5-dihydro-1H-imidazol-1-yl] benzamide (**72**). The compound (**72**) later upon reacted with thiourea yielded 4′-phenyl {[4′-(4′-benzylidene-5′-oxo-2-(pyridin-3-yl)-4, 5-dihydro-1H-imidazol-1-yl] (*p*-hydroxybenzoyl) amino} methyl carbamimidothioate (**73**). The compound (**73**) in turn reacted with diethyl malonate yielded N-{1-[(4", 6"-dioxo-tetrahydropyrimidin-2-yl) sulfanyl] benzyl}-N-[4-*p*-hydroxybenzylidene-5′-oxo-2-(pyridin-3-yl)-4, 5-dihydro-1H-imidazol-1-yl-benzamide (**74**).



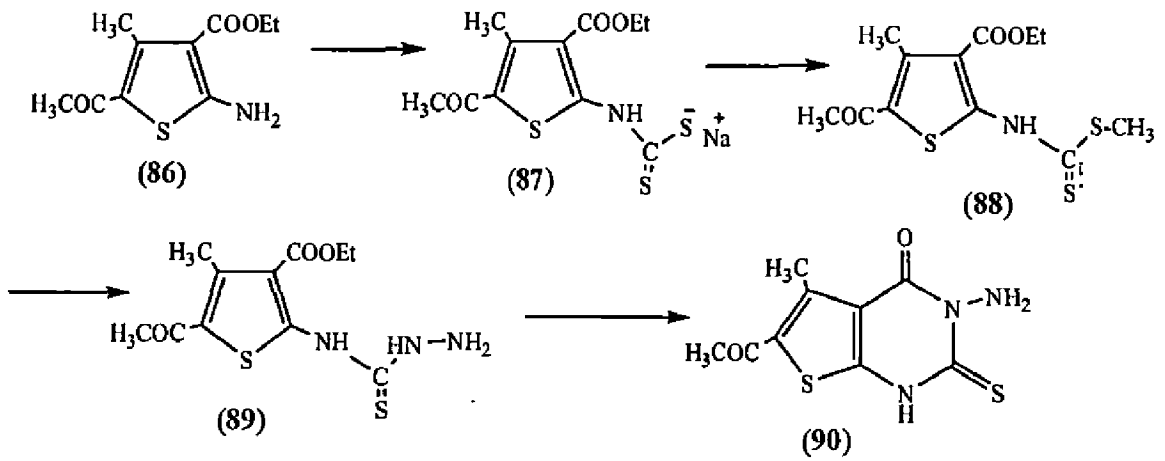
Drea and co-workers[30] reported the reaction of 5-amino-3-mercaptopyrazole (**75**) with 4-dimethylaminobenzaldehyde (**76**) which gave 5-(4′-dimethylamino) benzylidene) amino-3-mercaptopyrazole (**77**). The compound (**77**) on reaction with benzoyl chloride provided 5-(4′-dimethylamino)-chlorobenzylidene) N-(benzoyl) amino-3-mercaptopyrazole (**78**). The compound (**78**) reacted with guanidine hydrochloride and yielded 5-{(4′-dimethylamino)-benzylidene} N-(benzoyl) N-(guanidino)} amino-3-mercaptopyrazole (**79**) which later on reaction with diethyl malonate gave 5-{(4′-dimethylamino)-benzylidene) N-(benzoyl) (4, 6-dioxotetrahydropyrimidin-2-yl amino)-methyl)} amino-3-mercaptopyrazole (**80**).



Hasanen[31] reported the reaction of 3-acetylcoumarin (**81**) with thiosemicarbazide that provided 3-acetylcoumarin thiosemicarbazone (**82**) which reacted with diethyl malonate, chloro ethylacetate and ethyl acetoacetate to yield corresponding 4, 6-dihydroxy-1-(coumarin-3-ylethylidene) aminopyrimidin-2-thione (**83**), 3-(coumarin-3-ylethylidene) amino-2-thioxo-imidazolidin-4-one (**84**) and 6-methyl-1-(coumarin-3-ylethylidene) amino-2-thioxo-pyrimidin-4-one (**85**) in better yields.



Hafez and co-workers[32] reported the reaction of 5-acetyl-3-ethyl (2-amino-4-methyl thiophene) carboxylate (**86**) with CS_2 which gave methyl N-(4-methyl-5-acetyl-3-carboxyethyl-thiophene) dithiocarbamate (**88**). The compound (**88**) reacted with hydrazine and yielded methyl N-(4-methyl-5-acetyl-3-carboxyethyl-thiophene) thiosemicarbazide (**89**). The compound (**89**) in warmed ethanolic sodium hydroxide solution cyclized and gave 3-amino-6-acetyl-5-methyl-2-thioxo-thieno [2, 3 - *d*] pyrimidin-4-one (**90**).

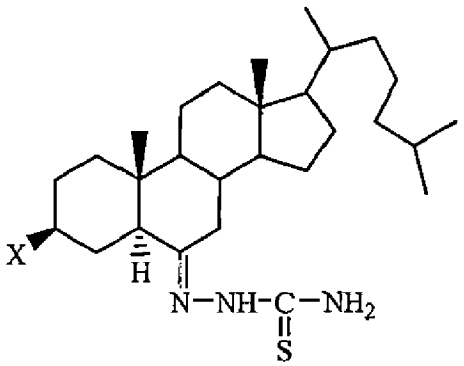


Discussion

Heterosteroids have been accredited with a great amount of attention over the years by medicinal chemists for drug discovery. The interesting structural and stereochemical features of the steroid nucleus provide additional fascination to the researchers and thereby alterations in the steroidal skeleton have been envisaged to discover new chemical entities with a potential to afford some promising drugs of the future. The incorporation of a heterocyclic ring like pyrimidine, thiazole or a heteroatom in the steroid backbone affects the chemical properties of a steroid and often results in useful alterations in its biological activities.[33] Therefore, researchers are on a continuous pursuit to design and produce better heterosteroids, by following natural models.

From last few years, numerous molecules possessing pyrimidine moiety have been reported to exhibit a broad spectrum of biological activities such as anticancer, antiviral, antibacterial, antioxidant, anxiolytic and antidepressant. Furthermore, they possess anti-inflammatory [34-42] and analgesic activities which are well documented in the literature.[43, 44] Besides this, pyrimidine derivatives have been explored for use as histamine and adenosine receptor antagonists as well as among several other biological receptors and modulators.[45]

The biological importance of these steroidal pyrimidines[34-45] and study of interesting behavior of diethyl malonate and ethyl cyanoacetate with simple thiosemicarbazones giving pyrimidines encouraged us to make similar studies with steroidal thiosemicarbazones. The substrates selected for synthesizing the new steroidal pyrimidines include 5α-cholestan-6-one thiosemicarbazone (**91**)[46], 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**92**)[46] and 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**93**).[46] The products obtained have been characterized on the basis of spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and elemental analyses.

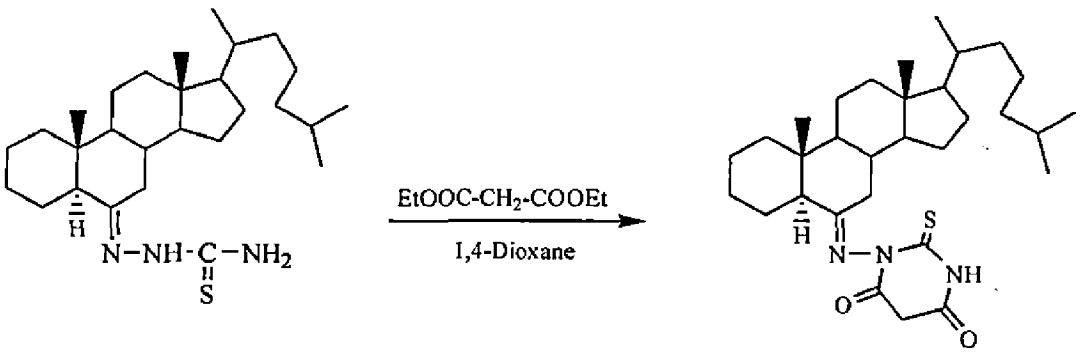


X

(**91**) H
(**92**) OAc
(**93**) Cl

Reaction of 5α-cholestan-6-one thiosemicarbazone (91) with diethyl malonate.

The 5α-cholestan-6-one thiosemicarbazone (**91**) in 1, 4-dioxane was allowed to react with diethyl malonate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to give compound **94**, m.p. 134 °C.



(**91**) (**94**)

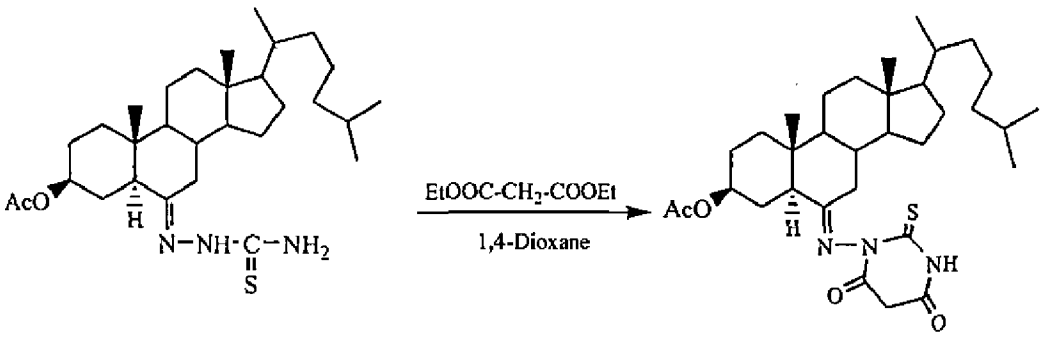
Characterization of the compound, m.p. 134 °C as [4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (94):

The elemental analysis of compound **94** corresponded to the molecular formula $C_{31}H_{49}N_3O_2S$. Its IR spectrum showed a band at 3322 cm^{-1} which could be assigned to NH group while as the bands at 1677, 1670, 1651, 1232 and 1025 cm^{-1} were attributed to *CO*NH, *CO*N, C=N, C=S and C-N group, respectively. These values supported the presence of pyrimidine moiety[47] in the product molecule. The structure **94** was well supported by its ^1H NMR spectrum which displayed a singlet integrating for one proton at δ 7.6 (exchangeable with D_2O) indicating the presence of N*H* while as another singlet integrating for two protons at δ 4.0 showed the presence of C_5'-H_2. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.22, 0.97, 0.98 and 0.74. The ^{13}C NMR spectrum of compound **94** displayed characteristic signals at δ 182 (C_2'), 172.1 (C_4'), 171.2 (C_6'), 153.3 (C_6), 52.2 (C_5') and 26 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **94** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 527) was found.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 1.1**), this compound can be best characterized as [4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**94**).

Reaction of 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (92) with diethyl malonate.

The 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (92) in 1, 4-dioxane was allowed to react with diethyl malonate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **95**, m.p. 167 °C.



(92) (95)

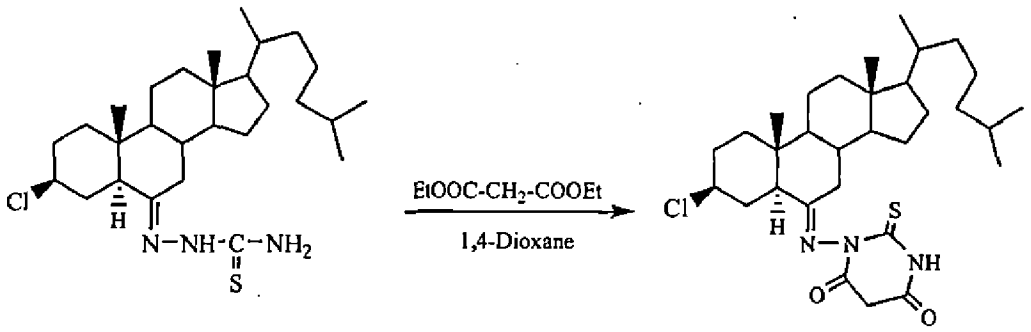
Characterization of the compound, m.p. 167 °C as 3β-acetoxy-[4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (95):

The compound **95** was correctly analyzed for the molecular formula $C_{33}H_{51}N_3O_4S$. Its IR spectrum showed a band at 3342 cm^{-1} which could be assigned to NH group. The strong absorption bands at 1734 and 1080 cm^{-1} indicated the presence of acetate group, while as the bands at 1679, 1674, 1643, 1269 and 1025 cm^{-1} were attributed to *CO*NH, *CO*N, C=N, C=S and C-N group, respectively. These values supported the presence of pyrimidine moiety[47] in the product molecule. The structure **95** was well supported by its ^1H NMR spectrum which displayed the singlet integrating for one proton at δ 8.3 (exchangeable with D_2O) indicating the presence of N*H* while as the singlet integrating for two protons at δ 4.2 showed the presence of C_5'-H_2. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. Three acetoxy group protons appeared at δ 2.04 as a sharp singlet. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.22, 0.97, 0.98 and 0.74. The ^{13}C NMR spectrum of compound **95** displayed characteristic signals at δ 183 (C_2'), 176.1 ($OCOCH_3$), 171.6 (C_4'), 170.2 (C_6'), 158.3 (C_6), 52.2 (C_5') and 70.4 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **95** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 585) was found.

On the basis of above studies and its analogy with earlier compound **94**, this compound can be best characterized as 3*β*-acetoxy-[4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5*α*-cholestane (**95**).

Reaction of 3*β*-chloro-5*α*-cholestan-6-one thiosemicarbazone (93) with diethyl malonate.

The 3*β*-chloro-5*α*-cholestan-6-one thiosemicarbazone (**93**) in 1, 4-dioxane was allowed to react with diethyl malonate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **96**, m.p. 151 °C.



(**93**) (**96**)

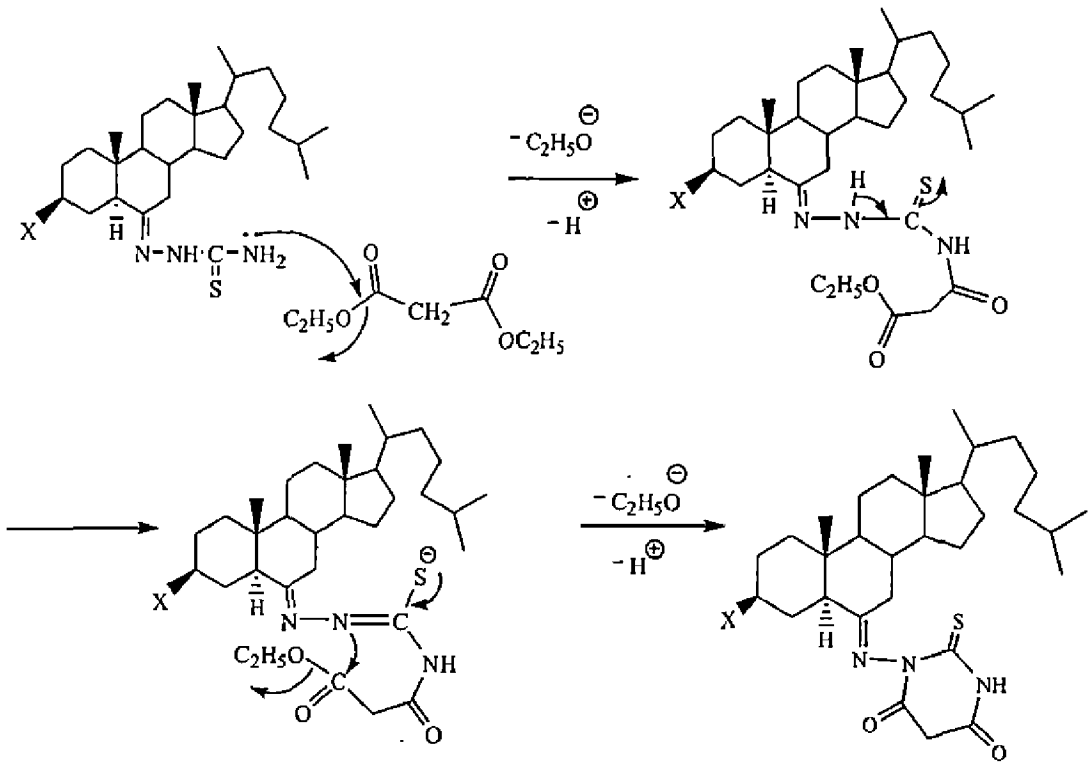
Characterization of the compound, m.p. 151 °C as 3*β*-chloro-[4′, 6′-dioxo-2′-thioxo-1H-pyrimidin-1-yl] 6-imino-5*α*-cholestane (96):

The compound **96** was correctly analyzed for the molecular formula $C_{31}H_{48}ClN_3O_2S$ (Beilstein positive). Its IR spectrum showed a band at 3315 cm^{-1} which could be assigned to NH group while as the bands at 1675, 1671, 1637, 1254, 1025 and 740 cm^{-1} were attributed to *CO*NH, *CO*N, C=N, C=S, C-N and C-Cl group, respectively. These values suggested the presence of pyrimidine moiety[47] in the product molecule. The structure **96** was well supported by its ^1H NMR spectrum which displayed the singlet integrating for one proton at δ 7.9 (exchangeable with D_2O) indicating the presence of N*H* while as the singlet integrating for two protons at δ 4.0 showed the presence of C_5'-H_2. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.22, 0.97, 0.98 and 0.74. The ^{13}C NMR spectrum of compound **96** displayed characteristic signals at δ 184 (C_2'), 172.1 (C_4'), 171.2 (C_6'), 157.3 (C_6), 59.6 (C_5') and 52.2 (C_3). Remaining carbon atoms were seen in

accordance to the cholestane series. The structure of compound **96** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 561/563) was observed.

The above data led to the structure of compound **96** as, 3β-chloro-[4', 6'-dioxo-2'-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane.

Formation of steroidal pyrimidines (**94-96**) under the condition case and in the light of available literature[10, 13, 15, 16, 22] may be shown according to the proposed mechanism (**Scheme 1.1**).



X

(**94**) H
(**95**) OAc
(**96**) Cl

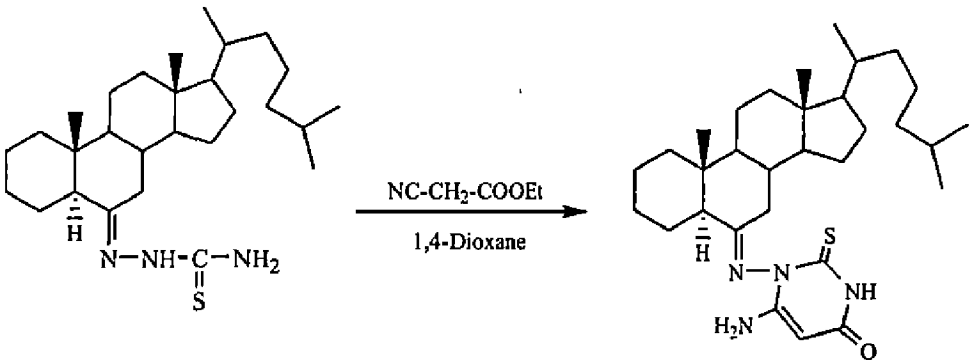
Scheme 1.1. Mechanism for the formation of [4',6'-dioxo-2'-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane derivatives (**94-96**)

Work published;

Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Molecular Structure* 1045 (2013) 62-71

Reaction of 5α-cholestan-6-one thiosemicarbazone (91) with ethyl cyanoacetate.

The 5α-cholestan-6-one thiosemicarbazone (91) in 1, 4-dioxane was allowed to react with ethyl cyanoacetate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to give compound **97**, m.p. 123 °C.



(91) (97)

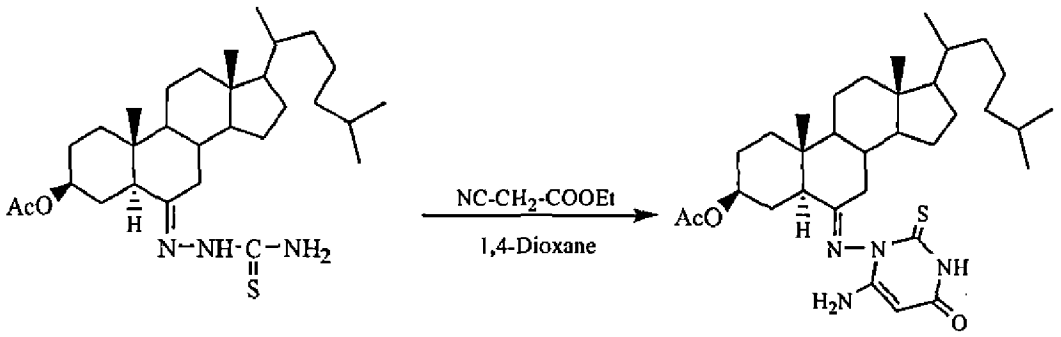
Characterization of the compound, m.p. 123 °C as [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (97):

The elemental analysis of compound **97** corresponded to the molecular formula $C_{31}H_{50}N_4OS$. Its IR spectrum showed bands at 3349 and 3284 cm^{-1} which could be assigned to NH and NH_2 groups while as the bands at 1670, 1653, 1620, 1264 and 1027 cm^{-1} were attributed to C=O, C=N, C=C, C=S and C-N group, respectively. These values supported the presence of pyrimidine moiety[47] in the product molecule. The structure **97** was well supported by its ^1H NMR spectrum which showed a singlet at δ 7.8 (exchangeable with D_2O) indicating the presence of N*H* while as a broad singlet at δ 3.2 (exchangeable with D_2O) integrating for two protons (NH_2). Another singlet for one proton appeared at δ 5.6 depicting the presence of an olefinic proton (C_5'-*H*) in the compound. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.17, 0.98, 0.94 and 0.72. The ^{13}C NMR spectrum of compound **97** displayed characteristic signals at δ 185 (C_2'), 173.6 (C_6'), 167.3 (C_4'), 154 (C_6), 81.4 (C_5') and 26 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **97** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 526) was observed.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 1.2**), this compound can be best characterized as [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**97**).

Reaction of 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (92) with ethyl cyanoacetate.

The 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**92**) in 1, 4-dioxane was allowed to react with ethyl cyanoacetate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **98**, m.p. 154 °C.



(92) (98)

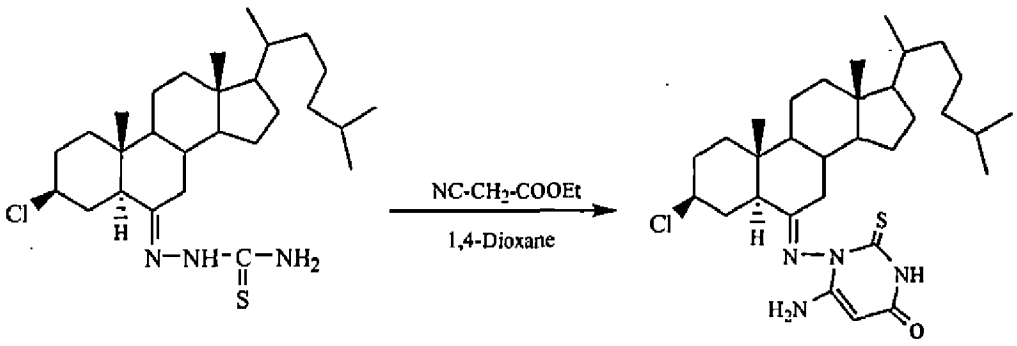
Characterization of the compound, m.p. 154 °C as 3β-acetoxy-[6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (98):

The compound **98** was correctly analyzed for the molecular formula $C_{33}H_{52}N_4O_3S$. Its IR spectrum showed bands at 3345 and 3290 cm^{-1} which could be assigned to NH and NH_2 groups. The strong absorption bands at 1714 and 1080 cm^{-1} indicated the presence of acetate group, while as the bands at 1671, 1645, 1622, 1269 and 1025 cm^{-1} were attributed to C=O, C=N, C=C, C=S and C-N group, respectively. These values suggested the presence of pyrimidine moiety[47] in the product molecule. The structure **98** was well supported by its ^1H NMR spectrum which showed a singlet at δ 8.2 (exchangeable with D_2O) indicating the presence of N*H* while as a broad singlet at δ 2.9 (exchangeable with D_2O) integrating for two protons (NH_2). Another singlet for one proton appeared at δ 5.7 depicting the presence of an olefinic proton (C_5'-*H*) in the compound. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. Three acetoxy group protons appeared at δ 2.03 as a sharp singlet. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound **98** displayed characteristic signals at δ 185 (C_2'), 173.2 (C_6'), 171.6 ($OCOCH_3$), 168.3 (C_4'), 155.3 (C_6), 85.8 (C_5') and 70 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **98** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 584) was found.

On the basis of above studies and its analogy with earlier compound **97**, this compound can be best characterized as 3β-acetoxy-[6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (**98**).

Reaction of 3β-chloro-5α-cholestan-6-one thiosemicarbazone (93) with ethyl cyanoacetate.

The 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**93**) in 1, 4-dioxane was allowed to react with ethyl cyanoacetate. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **99**, m.p. 138 °C.



(93) (99)

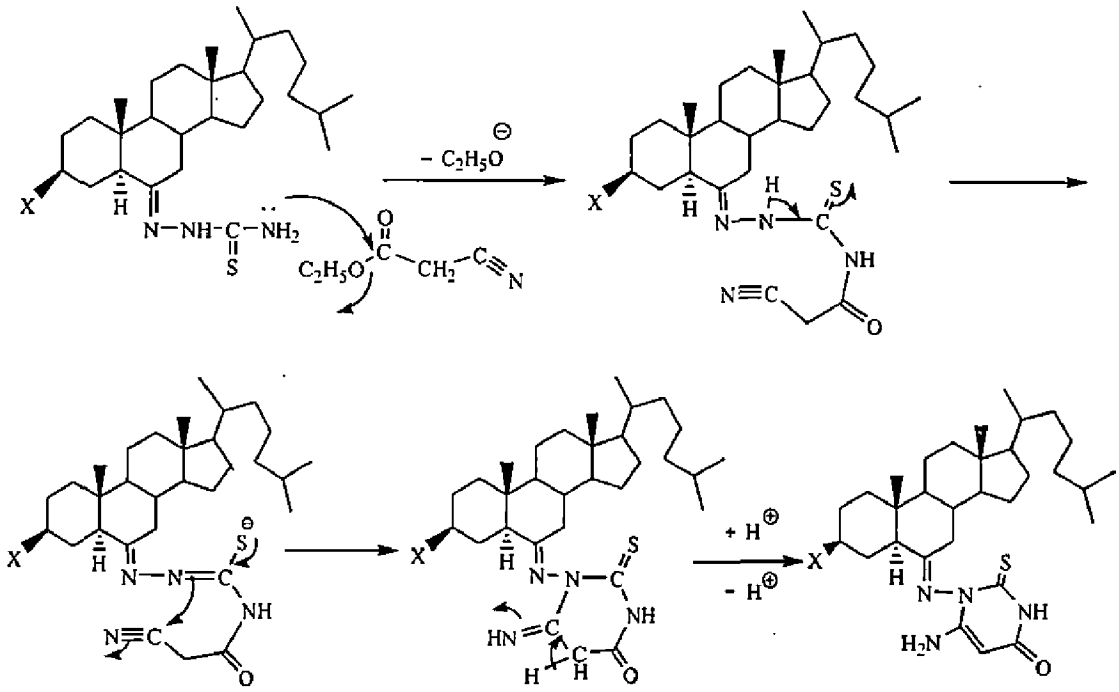
Characterization of the compound, m.p. 138 °C as 3β-chloro-[6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (99):

The compound **99** was correctly analyzed for the molecular formula $C_{31}H_{49}ClN_4OS$ (Beilstein positive). Its IR spectrum showed bands at 3353 and 3286 cm^{-1} which could be assigned to NH and NH_2 groups while as the bands at 1669, 1654, 1618, 1251, 1019 and 742 cm^{-1} were attributed to C=O, C=N, C=C, C=S, C-N and C-Cl group, respectively. These values supported the presence of pyrimidine moiety[47] in the product molecule. The structure **99** was well supported by its ^1H NMR spectrum which showed a singlet at δ 7.6 (exchangeable with D_2O) indicating one proton (N*H*) while as a broad singlet at δ 3.1 (exchangeable with D_2O) integrating for two protons (NH_2). Another singlet for one proton appeared at δ 5.5 depicting the presence of an olefinic proton (C_5'-*H*) in the compound. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to ($C_3\alpha$-*H*). The peaks for angular and side-chain methyl protons were observed at δ 1.17, 0.96, 0.84 and 0.72. The ^{13}C NMR spectrum of compound **99** displayed characteristic signals at δ 183 (C_2'), 171.6 (C_6'), 168.3 (C_4'), 155.3 (C_6), 79.8 (C_5') and 52.6 (C_3). Remaining

carbon atoms were seen in accordance to the cholestane series. The structure of compound **99** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 560/562) was observed.

The above data led to the structure of compound **99** as, 3*β*-chloro-[6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5*α*-cholestane.

Formation of steroidal pyrimidines (**97-99**) under the condition case and in the light of available literature[22, 26] may be shown according to the proposed mechanism (**Scheme 1.2**).



X

(**97**) H
(**98**) OAc
(**99**) Cl

Scheme 1.2. Mechanism for the formation of [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5*α*-cholestane derivatives (**97-99**)

Work published;

DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1H-pyrimidines, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Comptes Rendus Chimie*, http://dx.doi.org/10.1016/j.crci.2013.07.001 (in press)

Experimental

All the melting points were determined in degrees Celsius on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Perkin Elmer RXI Spectrophotometer and values are given in cm^{-1}. ^1H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of boiling point 60-80 °C. Sodium sulfate (anhydrous) was used as a drying agent.

3β-Chlorocholest-5-ene:

Freshly purified thionyl chloride (40 mL) was added gradually to cholesterol (50 g) at room temperature. A vigorous reaction ensured with evolution of gaseous products. When the reaction slackened, the mixture was gently heated at temperature 50-60 °C on water bath for 1 h and then poured into crushed ice-water with stirring. The yellow solid thus obtained was filtered under suction and washed several times with ice-cold water and air dried. Recrystallization of crude product from acetone gave 3β-chlorocholest-5-ene (42 g), m.p. 95-96 °C (reported m.p. 96-97 °C).[48] It gave positive Beilstein test and a yellow color with tetra-nitromethane in chloroform.

Cholest-5-ene:

3β-Chlorocholest-5-ene (10 g) was dissolved in warm amyl alcohol (230 mL) and sodium metal (20 g) was added in small portions to the solution with continuous stirring over the period of 8 h. The reaction mixture was warmed occasionally. When all the sodium metal was dissolved, the reaction mixture was poured into water, acidified with dilute hydrochloric acid and allowed to stand overnight. A white crystalline solid thus obtained was filtered under suction, washed thoroughly with water and air dried. Recrystallization of the crude material from acetone gave cholest-5-ene (8.3 g) in cubes, m.p. 92 °C (reported m.p. 89-91 °C).[49]

6-Nitrocholest-5-ene:

A suspension of finely powdered cholest-5-ene (6 g) in glacial acetic acid (50 mL) was stirred at room temperature for 5 min. and nitric acid (15 mL; d, 1.52 g/cm^3) was rapidly added followed by the addition of sodium nitrite (3 g) and stirring was continued for further 2 h. The temperature of a reaction mixture was controlled between 20-25 °C by external cooling. The reaction mixture was then poured into cold water and the yellow solid thus

obtained was filtered under suction, washed thoroughly with water and air dried. Recrystallization of the crude material from methanol furnished 6-nitrocholest-5-ene (4.5 g), m.p. 119-120 °C (reported m.p. 120-121 °C).[50]

5α-Cholestan-6-one:

6-Nitrocholest-5-ene (6 g) was dissolved in glacial acetic acid (120 mL) by heating and to this solution; zinc dust (12 g) was gradually added in small portions with shaking. After the initial exothermic reaction was subsided, the suspension was heated under reflux for 4 h and water (12 mL) was added during the course of reaction. The solution was then filtered and the residue was washed with two (10 mL) portions of warm acetic acid. To the filtrate, a few mL of water was added till turbidity developed and it was allowed to stand overnight at room temperature. The crystalline material thus separated was filtered under suction and washed thoroughly with water in order to remove zinc acetate. The organic solid was air dried and then recrystallized from methanol (3.6 g), m.p. 96-98 °C (reported m.p. 98-100 °C).[48]

5α-Cholestan-6-one thiosemicarbazone (91):

To a boiling solution of 5α-cholestan-6-one (3.85 g) in ethanol (20 mL), a few drops of concentrated hydrochloric acid were added followed by the addition of solution of thiosemicarbazide (1.01 g) in ethanol (10 mL) with stirring. The reaction mixture was refluxed for 1 h and then cooled. The precipitate thus obtained was separated by filtration and purified by recrystallization from methanol to give 5α-cholestan-6-one thiosemicarbazone (**91**) (3.2 g) as shinning needles, m.p. 112-114 °C (reported m.p. 113 °C).[46]

3β-Acetoxycholest-5-ene:

A mixture of cholesterol (50 g), pyridine (75 mL freshly distilled over KOH) and freshly distilled acetic anhydride (50 mL) was heated on a water bath for 2 h. The resulting brown colored reaction mixture was poured into crushed ice-water mixture with stirring. A light brown solid thus obtained was filtered under suction, washed with water until free from pyridine and air dried. The crude product on crystallization from acetone gave pure 3β-acetoxycholest-5-ene (45 g), m.p. 113-114 °C (reported m.p. 115-116 °C).[51]

3β-Acetoxy-6-nitrocholest-5-ene:

To a cooled mixture of 3β-acetoxycholest-5-ene (10 g) and nitric acid (250 mL; d, 1.42 g/cm^3), sodium nitrite (10 g) was gradually added over a period of 45 min. with constant stirring. Slight cooling was also affecting during the course of reaction and stirring was

continued for additional 2 h and then cold water (350 mL) was added to reaction mixture. The yellow solid material thus separated was extracted with diethyl ether and the ethereal layer was washed with water, sodium bicarbonate solution (5%) (until washing was pink), again with water and finally dried by anhydrous sodium sulfate. Removal of solvents provided the compound as an oil which was crystallized from methanol (40 mL) to give 3β-acetoxy-6-nitrocholest-5-ene (7.0 g), m.p. 104 °C (reported m.p. 102-104 °C).[52]

3β-Acetoxy-5α-cholestan-6-one:

3β-Acetoxy-6-nitrocholest-5-ene (6 g) was dissolved in hot glacial acetic acid (250 mL) and zinc dust (12 g) was added in small portions with shaking. The suspension was heated under reflux for 4 h and water (12 mL) was added during the course of reaction. Zinc dust was removed by filtration and the filtrate was diluted with large excess of water. Usual work up of the reaction mixture afforded the ketone, which was crystallized from methanol (3.9 g), m.p. 128 °C (reported m.p. 127-128 °C).[53]

3β-Acetoxy-5α-cholestan-6-one thiosemicarbazone (92):

To a boiling solution of 3β-acetoxy-5α-cholestan-6-one (4.44 g) in ethanol (20 mL), a few drops of concentrated hydrochloric acid were added followed by the addition of solution of thiosemicarbazide (1.01 g) in ethanol (10 mL) with stirring. The reaction mixture was refluxed for 1 h and then cooled. The precipitate was separated by filtration and purified by recrystallization from methanol to give 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**92**) as shinning needles (3.3 g), m.p. 128-129 °C (reported m.p. 128 °C).[46]

3β-Chloro-6-nitrocholest-5-ene:

To a stirred mixture of 3β-chlorocholest-5-ene (14 g), glacial acetic acid (100 mL) and nitric acid (28 mL; d, 1.52 g/cm^3) at temperature below 20 °C was added sodium nitrite (3.5 g) gradually added over a period of 1 h. After complete addition of sodium nitrite, the reaction mixture was stirred for an additional period of 2 h. Ice cold water (200 mL) was added and the yellowish solid separated was filtered and air dried. The desired product was crystallized from methanol as needles (8.9 g), m.p. 151-152 °C (reported m.p. 153 °C).[54]

3β-Chloro-5α-cholestan-6-one:

A solution of 3β-chloro-6-nitrocholest-5-ene (12 g) and glacial acetic acid (240 mL) was heated to get a clear solution. The zinc dust (24 g) was added gradually in small portions with constant shaking. The suspension was heated under reflux for 4 h and water (24 mL) was

added at regular intervals during the course of reaction. The hot solution was poured into ice cold water. The organic matter was extracted with diethyl ether and the ethereal layer was successively washed with water, sodium bicarbonate solution (5 %) and again with water and dried over anhydrous sodium sulfate. Evaporation of the solvent furnished the product as an oil which was crystallized from methanol (8.7 g), m.p. 128-129 °C (reported m.p. 129 °C).[55]

3β-Chloro-5α-cholestan-6-one thiosemicarbazone (93):

To a boiling solution of 3β-chloro-5α-cholestan-6-one (4.2 g) in ethanol (20 mL), few drops of concentrated hydrochloric acid were added followed by the addition of solution of thiosemicarbazide (1.01g) in ethanol (10 mL) with stirring. The reaction mixture was refluxed for 1 h and cooled. The precipitate thus obtained was separated by filtration and purified by recrystallization from methanol to give 3β-chloro-5α-cholestan-6-one thiosemicarbazone (**93**) as crystalline solid (3.3 g), m.p. 135-136 °C (reported m.p. 136 °C).[46]

Reaction of 5α-cholestan-6-one thiosemicarbazone derivatives (91-93) with diethyl malonate/ethyl cyanoacetate:

To a solution of 5α-cholestan-6-one thiosemicarbazone derivatives (**91-93**) (1.5 mmol) in 1, 4-dioxane (20 mL), an equimolar amount of diethyl malonate/ ethyl cyanoacetate was added. The reaction mixture was refluxed for 10-11 h. The progress and completion of the reaction was monitored by TLC. After completion of reaction, the excess solvent was reduced to three fourths of the original volume under reduced pressure. The reaction mixture was then taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Evaporation of solvents and crystallization of the oily residue from methanol afforded the corresponding products (**94-99**).

[4', 6'-Dioxo-2'-thioxo-1H-pyrimidin-1-yl]6-imino-5α-cholestane (94):

Yield 70%; m.p. 134 °C; Analysis found: C 70.58, H 9.29, N 7.96%. $C_{31}H_{49}N_3O_2S$ requires: C 70.54, H 9.35, N 7.96%; IR (KBr): ν_{max} 3322 (NH), 1677 (*C*ONH), 1670 (*C*ON), 1651 (C=N), 1232 (C=S), 1025 (C-N); ^{1}H NMR ($CDCl_3$): δ 7.6 (s, 1H, N*H*, exchangeable with D_2O), 4.0 (s, 2H, C_5'-H_2), 1.22 (s, 3H, C_{10}-CH_3), 0.74 (s, 3H, C_{13}-CH_3), 0.97 and 0.98 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 182 (C_2'), 172.1 (C_4'), 171.2 (C_6'), 153.3 (C_6), 52.2 (C_5'), 48 (C_{17}), 46.7 (C_{14}), 42.1 (C_{10}), 40.1 (C_5), 37 (C_{20}), 35 (C_{18}), 26 (C_3), 24 (C_2), 22 (C_4), 20 (C_{16}); MS: *m/z* 527 [$M^{+\cdot}$].

3β-Acetoxy-[4', 6'-dioxo-2'-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane (95):

Yield 78%; m.p. 167 °C; Analysis found: C 67.69, H 8.71, N 7.17%. $C_{33}H_{51}N_3O_4S$ requires: C 67.65, H 8.77, N 7.17%; IR (KBr): ν_{max} 3342 (NH), 1734 ($OCOCH_3$), 1679 (*CO*NH), 1674 (*CO*N), 1643 (C=N), 1269 (C=S), 1080 (C-O), 1025 (C-N); ^{1}H NMR ($CDCl_3$): δ 8.3 (s, 1H, N*H*, exchangeable with D_2O), 4.7 (m, 1H, $C_3\alpha$-*H*, *W½* = 15 Hz), 4.2 (s, 2H, C_5'-H_2), 2.04 (s, 3H, $OCOCH_3$), 1.22 (s, 3H, C_{10}-CH_3), 0.74 (s, 3H, C_{13}-CH_3), 0.97 and 0.98 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 183 (C_2'), 176.1 (CH_3COO), 171.6 (C_4'), 170.2 (C_6'), 158.3 (C_6), 70.4 (C_3), 52.2 (C_5'), 48 (C_{17}), 46.7 (C_{14}), 42.1 (C_{10}), 40.1 (C_5), 37 (C_{20}), 35 (C_{18}), 24 (C_2), 22 (C_4), 20 (C_{16}); MS: *m/z* 585 [$M^{+\cdot}$].

3β-Chloro-[4', 6'-dioxo-2'-thioxo-1H-pyrimidin-1-yl]6-imino-5α-cholestane (96):

Yield 80%; m.p. 151 °C; Analysis found: C 66.31, H 8.55, N 7.48%. $C_{31}H_{48}ClN_3O_2S$ requires: C 66.22, H 8.60, N 7.47%; IR (KBr): ν_{max} 3315 (NH), 1675 (*CO*NH), 1671 (*CO*N), 1637 (C=N), 1254 (C=S), 1025 (C-N), 740 (C-Cl); ^{1}H NMR ($CDCl_3$): δ 7.9 (s, 1H, N*H*, exchangeable with D_2O), 4.0 (s, 2H, C_5'-H_2), 3.9 (m, 1H, $C_3\alpha$-*H*, *W½* = 17 Hz), 1.22 (s, 3H, C_{10}-CH_3), 0.74 (s, 3H, C_{13}-CH_3), 0.97 and 0.98 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 184 (C_2'), 172.1 (C_4'), 171.2 (C_6'), 157.3 (C_6), 59.6 (C_5'), 52.2 (C_3), 46.7 (C_{14}), 42.1 (C_{10}), 40.1 (C_5), 37 (C_{20}), 35 (C_{18}), 24 (C_2), 22 (C_4), 20 (C_{16}); MS: *m/z* 561/563 [$M^{+\cdot}$].

[6'-Amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl]6-imino-5α-cholestane (97):

Yield 70%; m.p. 123 °C; Analysis found: C 70.66, H 9.45, N 10.61%. $C_{31}H_{50}N_4OS$ requires: C 70.57, H 9.39, N 10.58%; IR (KBr): ν_{max} 3349, 3284 (NH, NH_2), 1670 (C=O), 1653 (C=N), 1620 (C=C), 1264 (C=S), 1027 (C-N); ^{1}H NMR ($CDCl_3$): δ 7.8 (s, 1H, N*H*, exchangeable with D_2O), 5.6 (s, 1H, C_5'-*H*), 3.2 (s, 2H, NH_2, exchangeable with D_2O) 1.17 (s, 3H, C_{10}-CH_3), 0.72 (s, 3H, C_{13}-CH_3), 0.98 and 0.94 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 185 (C_2'), 173.6 (C_6'), 167.3 (C_4'), 154.3 (C_6), 81.4 (C_5'), 39 (C_5), 35 (C_{10}), 26 (C_3), 24 (C_7), 22 (C_{16}), 20 (C_{15}); MS: *m/z* 526 [$M^{+\cdot}$].

3β-Acetoxy-[6'-amino-2'-thioxo-4'-oxodihydro-1H-pyrimidin-1-yl]6-imino-5α-cholestane (98):

Yield 80%; m.p. 154 °C; Analysis found: C 67.80, H 8.90, N 9.53%. $C_{33}H_{52}N_4O_3S$ requires: C 67.69, H 8.84, N 9.49%; IR (KBr): ν_{max} 3345, 3290 (NH, NH_2), 1714 ($OCOCH_3$), 1671 (C=O), 1645 (C=N), 1622 (C=C), 1269 (C=S), 1080 (C-O), 1025 (C-N); ^{1}H NMR ($CDCl_3$): δ 8.2 (s, 1H, N*H*, exchangeable with D_2O), 5.7 (s, 1H, C_5'-*H*), 4.7 (m, 1H, $C_3\alpha$-*H*, *W½* =15 Hz), 2.9 (s, 2H, NH_2, exchangeable with D_2O), 2.03 (s, 3H, $OCOCH_3$), 1.18 (s, 3H, C_{10}-CH_3), 0.70

(s, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 185 (C_2'), 173.2 (C_6'), 171.6 (O*C*OCH$_3$), 168.3 (C_4'), 155.3 (C_6), 85.8 (C_5'), 70 (C_3), 39 (C_5), 35 (C_{10}), 24 (C_7), 22 (C_{16}), 20 (C_{15}); MS: *m/z* 584 [$M^{+\cdot}$].

3β-Chloro-[6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl]6-imino-5α-cholestane (99):

Yield 75%; m.p. 138 °C; Analysis found: C 66.42, H 8.75, N 9.9%. $C_{31}H_{49}ClN_4OS$ requires: C 66.34, H 8.64, N 9.87%; IR (KBr): ν_{max} 3353, 3286 (NH, NH_2), 1669 (C=O), 1654 (C=N), 1618 (C=C), 1251 (C=S), 1019 (C-N), 742 (C-Cl); ^{1}H NMR ($CDCl_3$): δ 7.6 (s, 1H, N*H*, exchangeable with D_2O), 5.5 (s, 1H, C_5'-*H*), 3.9 (m, 1H, C_3*α-H*, *W* ½ = 17 Hz), 3.1 (s, 2H, NH_2, exchangeable with D_2O) 1.17 (s, 3H, C_{10}-CH_3), 0.72 (s, 3H, C_{13}-CH_3), 0.96 and 0.84 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 183 (C_2'), 171.6 (C_6'), 168.3 (C_4'), 155.3 (C_6), 79.8 (C_5'), 52.6 (C_3), 39 (C_5), 35 (C_{10}), 24 (C_7), 22 (C_{16}), 20 (C_{15}); MS: *m/z* 560/562 [$M^{+\cdot}$].

References

References

1. J. A. Joule, K. Mills, *Heterocyclic Chemistry* (John Wiley & Sons) Oxford 5th Edn. p. 250, 2010
2. Lagoja, M. Irene, *Chemistry and Biodiversity*, **2**, 1 (2005)
3. M. E. Grimaux, *Proceedings weekly sessions of the Academy of Sciences*, **88**, 85 (1879)
4. A. Pinner, *Chem. Ber.*, **17**, 2519 (1884)
5. A. Pinner, *Chem. Ber.*, **18**, 759 (1885)
6. S. Gabriel, J. Colman, *Chem. Ber.*, **33**, 3666 (1900)
7. R. Behrend, *Ann. Chem.*, **229**, 18 (1885)
8. S. M. Hussain, A. A. El-Barbary, S. A. Mansour, *J. Heterocycl. Chem.*, **22**, 169 (1985)
9. M. A. Pere, J. L. Soto, F. J. Guzman, *J. Chem. Soc., Perkin Trans.*, **1**, 87 (1985)
10. E. C. Taylor, R. W. Morrison, *J. Org. Chem.*, **32**, 2379 (1967)
11. M. Botta, M. C. Dceci, F. D. Angelis, G. Finizia, R., Nicoletti, *Tetrahedron*, **40**, 3313 (1984)
12. Andereichikov, S. Yu. G. D. Plakhina, *Zh. Org. Khim.*, **23**, 872 (1987)
13. H. I. El-Subbagh, *Sulfur Lett.*, **11**, 249 (1990)
14. A. Bowman, *J. Chem. Soc.*, 494 (1937)
15. K. M. Yossef, R. H. Omar, M. El-Meleigy, *Zagazig J. Pharm. Sci.*, **3**, 182 (1994)
16. E. A. Mohamed, *Chem. Pap.*, **48**, 261 (1994)
17. F. A. Attaby, S. M. Eldin, *Arch. Pharm. Res.*, **20**, 330 (1997)
18. W. Stadlbauer, E. Badawey, G. Hojas, P. Roschger, T. Kappe, *Molecules*, **6**, 338 (2001)
19. D. G. Rivera, K. Peseke, I. Jomarrón, A. Montero, R. Molina, F. Coll, *Molecules*, **8**, 444 (2003)
20. B. B. Semenov, K. A. Novikov, K. A. Krasnov, V. V. Kachala, *Chem. Heterocycl. Comp.*, **41**, 5 (2005)
21. R. M. Abdel-Rahman, M. S. I.T. Makki, W. A. B. Bawazir, *E - J. Chem.*, **7**, 93 (2010)
22. B. Parashar, S. Bhardwaj, S. Sharma, G. D. Gupta, V. K. Sharma P. B. Punjabi, *J. Chem. Pharm. Res.*, **2**, 33 (2010)
23. D. F. Hassan, *J. Al-Nahrain Univer. Sci.*, **13**, 32 (2010)
24. Y.A. Ammar, Y. A. Mohamed, A. M. El-Sharief, M. S. A. El-Gaby, S. Y. Abbas, *Chem. Sci. J.*, CSJ-15 (2011)
25. G. Naganagowda, P. Thamyongkit, R. Klai-U-dom, W. Ariyakriangkrai, A. Luechai, A. Petsom, *J. Sulfur Chem.*, **32**, 235 (2011)

26. M. I. El-Zahari, S. S. Abdel-Karim, M. E. Haiba, M. A. Khedr, *Acta Poloniae Pharm. Drug Res.*, **68**, 357 (2011).

27. V. V. Dabholkar, R. P. Gavande, *Acta Poloniae Pharm. Drug Res.*, **69**, 247 (2012)

28. B. F. Abdel-Wahab, H. A. Mohamed, *Turk. J. Chem.*, **36**, 805 (2012)

29. A. J. K. Al. Abodi, N. Majed. , A. K. Sahar, R. I. H. Al-Bayati, *American J. Org. Chem.*, **2**, 143 (2012)

30. F. O. Essa, K. J. Kadhum, A. A. Drea, *J. App. Chem.*, **1**, 344 (2012)

31. J. A. Hasanen, *Der. Pharma. Chem.*, **4**, 1923 (2012)

32. H. N. Hafez, A. R. B. A. El-Gazzar, G. A.M. Nawwar, *Eur. J. Med. Chem.*, **45**, 1485 (2010)

33. H. Singh, V. K. Kapoor, D. Paul, *Heterosteroids and drug research. In: Progress in Medicinal Chemistry*, Ellis GP, West GB. Eds., Springer: Amsterdam, p. 35, 1979

34. Y. Deng, Y. Wang, C. Cherian, Z. Hou, S. A. Buck, L. H. Matherly, A. Gangjee, *J. Med. Chem.*, **51**, 5052 (2008)

35. T. L. Nguyen, *Anti-Cancer Agents Med. Chem.*, **8**, 710 (2008)

36. T. C. Renau, C. Kennedy, R. G. Ptak, J. M. Breitenbach, J. C. Drach, L. B. Townsend, *J. Med. Chem.*, **39**, 3470 (1996)

37. L. F. Kuyper, J. M. Garvey, D. P. Baccanari, J. N. Champness, D. K. Stammers, C. R. Beddell, *Bioorg. Med. Chem.*, **4**, 593 (1996)

38. P. K. Andrus, T. J. Fleck, J. A. Oostveen, E. D. Hall, *J. Neurosci. Res.*, **47**, 650 (1997)

39. G. L. Bundy, D. E. Ayer, L. S. Banitt, K. L. Belonga, S. A. Mizsak, J. R. Palmer, J. M. Tustin, J. E. Chin, E. D. Hall, *J. Med. Chem.*, **38**, 4161 (1995)

40. E. A. Meada, M. Sznaidman, G. T. Pollard, L. M. Beauchamp, J. L. Howard, *Eur. J. Med. Chem.*, **33**, 363 (1998)

41. V. Darias, S. Abdala, D. Martin-Herrera, S. Vega, *Arzneimittelforschung*, **49**, 986 (1999)

42. E. P. Das, Falcao, S. J. Melo, R. M. Srivastava, M. T. J. Catanho, S. C. D. Nascimento, *Eur. J. Med. Chem.* **41**, 276 (2006)

43. B. C. Bookser, B. G. Ugarkar, M. C. Matelich, R. H. Lemus, M. Allan, M. Tsuchiya, M. Nakane, A. Nagahisa, J. B. Wiesner, M. D. Erion, *J. Med. Chem.*, **48**, 7808 (2005)

44. S. M. Sondhi, N. Singh, M. Johar, A. Kumar, *Bioorg. Med. Chem.*, **13**, 6158 (2005)

45. R. J. Gillespie, S. J. Bamford, S. Gaur, A. M. Jordan, J. Lerpiniere, H. L. Mansell, G. C. Stratton, *Bioorg. Med. Chem. Lett.*, **19**, 2664 (2009)

46. Shamsuzzaman, M. Aslam, A. Salim, *J. Chem. Res.*, 824 (1998)

47. G. Socrates, *Infrared Charatcteristic Group Frequencies* (John-wiley & Sons), p. 102,

1980

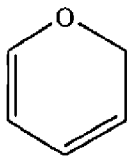
48. C. W. Shoppe, R. H. Jenkins, G. H. R. Summers, *J. Chem. Soc.*, 1657 (1958)
49. W. G. Douben, K. H. Takemura, *J. Am. Chem. Soc.*, **75**, 6302 (1953)
50. J. R. Bull, E. R. H. Jones, G. D. Meakins, *J. Chem. Soc.*, 2601 (1965)
51. L. F. Fieser, M. Fieser, "*Steroids*" Reinhold Publishing Corporation, New York, p. 28, 1959
52. C. E. Anagostopoulos, L. F. Fieser, *J. Am. Chem. Soc.*, **76**, 532 (1954)
53. R. M. Dodson, B. Riegel, *J. Org. Chem.*, **13**, 424 (1948)
54. R. H. Baker, E. N. Squire, *J. Am. Chem. Soc.*, **70**, 1487 (1948)
55. N. F. Blau, C. G. Stuckwisch, *J. Org. Chem.*, **21**, 370 (1962)

Chapter-2

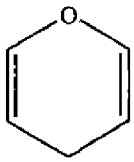
Synthesis of steroidal pyrans

Theoretical

Pyran is a six membered non-aromatic ring consisting of five carbon atoms, one oxygen atom and two double bonds. The molecular formula is C_5H_6O. The term pyran is also often applied to the saturated ring analog, which is more properly referred to as tetrahydropyran (oxane). There are two isomers of pyran that differ by the location of the double bonds. In 2H-pyran (**1**), the saturated carbon is at position 2 while as in 4H-pyran (**2**), the saturated carbon is at position 4.

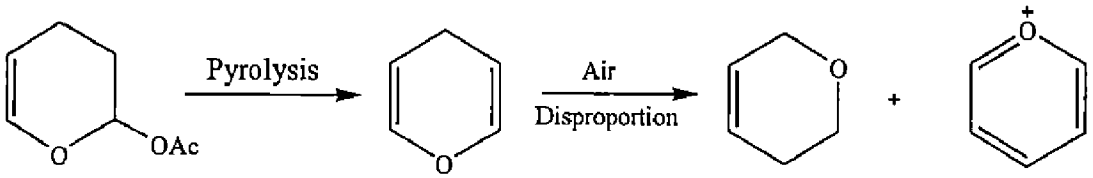


(**1**)



(**2**)

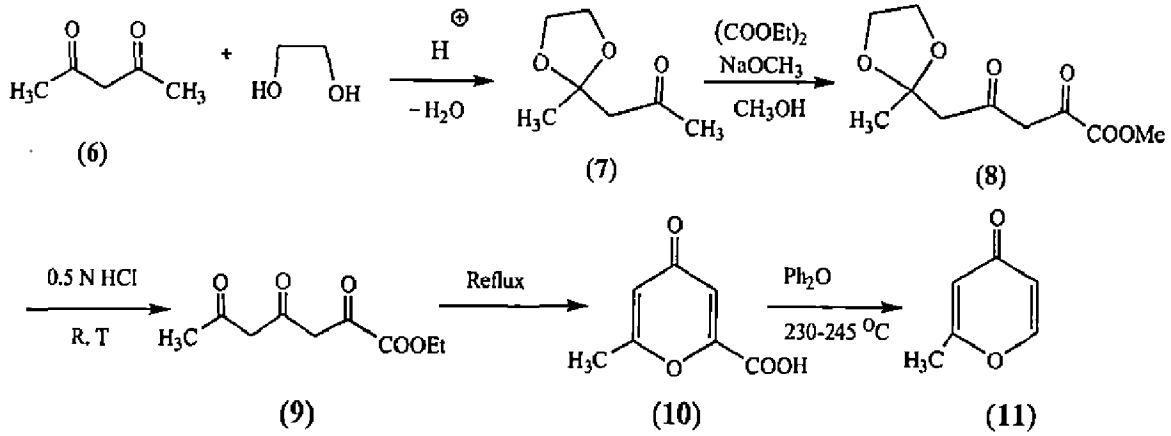
Masamune and Castellucci[1] in 1962 first isolated and characterized 4H-pyran by the pyrolysis of 2-acetoxy-3, 4-dihydro-2H-pyran (**3**). It was found too unstable, particularly in the presence of air and easily disproportionated to the corresponding dihydropyran (**4**) and the pyrylium ion (**5**).



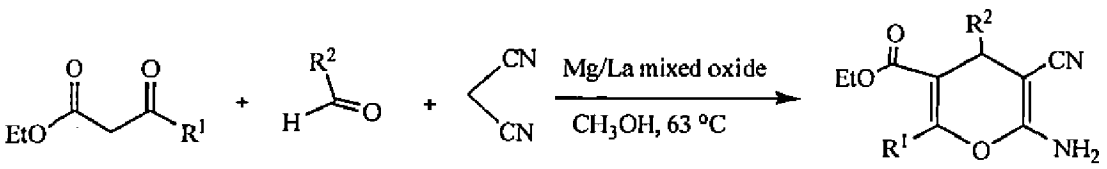
(**3**) (**2**) (**4**) (**5**)

Dorman[2] in 1967 reported the reaction of acetyl acetone (**6**) with ethylene glycol which gave 2-methyl-2-acetonyl-1, 3-dioxolan (**7**). The compound (**7**) was acylated with diethyl oxalate in presence of sodium methoxide formed methyl 5-(2-methyl-[1, 3] dioxolan-2-yl)-2, 4-dioxopentanoic acid methyl ester (**8**). The compound (**8**) was treated with 0.5 N HCl to give intermediate triketone (**9**). The compound (**9**) was refluxed to complete ring closure forming 6-methyl-4H-pyran-4-one-2-carboxylic acid (**10**) which on subsequent decarboxylation yielded 2-methyl-4H-pyran-4-one (**11**).

THESI



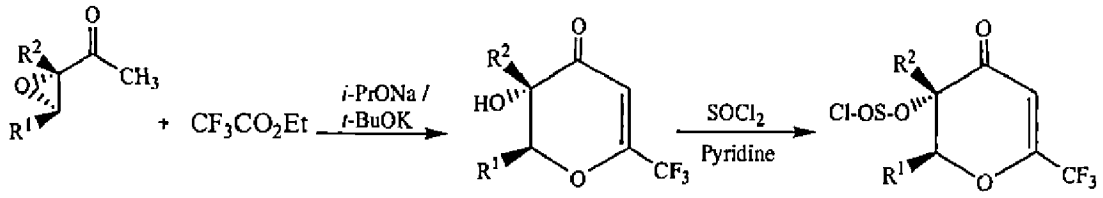
Lingaiah and co-workers[3] reported an efficient synthesis of polyfunctionalized 4H-pyrans (**14 a-h**) which involved one pot condensation of active methylenic diketo compounds (**12 a-h**), aldehydes (**13 a-h**) and malononitrile using basic Mg/La mixed oxide as catalyst.



(12) (13) (14)

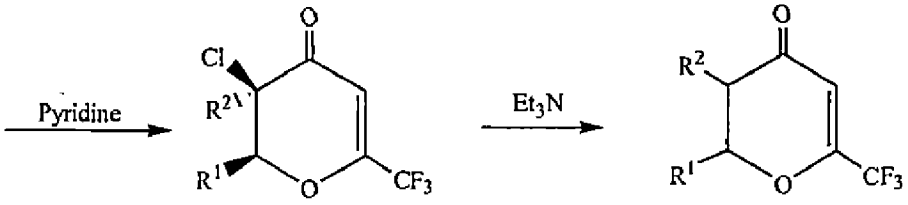
R^1	R^2	R^1	R^2
(a) C_2H_4Br	(a) C_6H_5	(a) C_2H_4Br	C_6H_5
(b) C_2H_5	(b) 4-NO_2-C_6H_4	(b) C_2H_5	4-NO_2-C_6H_4
(c) C_3H_7	(c) 3-NO_2-C_6H_4	(c) C_3H_7	3-NO_2-C_6H_4
(d) CH_2Cl	(d) 4-Cl-C_6H_4	(d) CH_2Cl	4-Cl-C_6H_4
(e) CH_2Br	(e) 4-CN-C_6H_4	(e) CH_2Br	4-CN-C_6H_4
(f) C_6H_4Cl	(f) 4-OH-C_6H_4	(f) C_6H_4Cl	4-OH-C_6H_4
(g) C_6H_4Br	(g) 4-Me-C_6H_4	(g) C_6H_4Br	4-Me-C_6H_4
(h) CH_2I	(h) 4-OMe-C_6H_4	(h) CH_2I	4-OMe-C_6H_4

Tyvorskii and co-workers[4] reported the reaction of oxiranes (**15 a-e**) with ethyl perfluoroalkanoate in presence of sodium *iso*-propoxide or potassium *tert*-butoxide that gave hydroxypyranones (**16 a-e**). The hydroxypyranones upon reaction with thionyl chloride in dry pyridine yielded chlorosulfites (**17 a-e**). These sulphites upon refluxing in presence of pyridine provided chlorosubstituted pyranones (**18 a-e**) which on reaction with triethyl amine yielded 2-perfluoroalkyl-4H-pyran-4-ones (**19 a-e**).



(15) (16) (17)

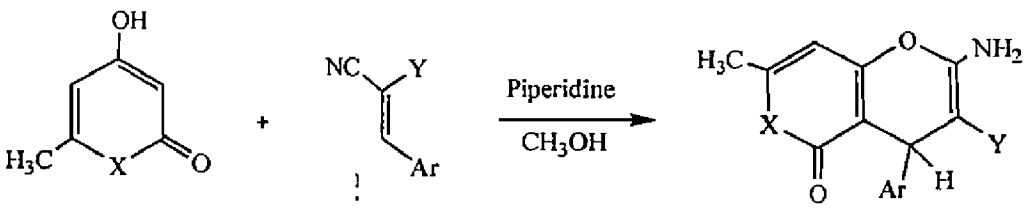
	R^1	R^2
(a)	H	CH_3
(b)	H	Ph
(c)	CH_3	CH_3
(d)	CH_2Cl	CH_3
(e)	CH_2I	Ph



(18) (19)

	R^1	R^2
(a)	H	CH_3
(b)	H	Ph
(c)	CH_3	CH_3
(d)	CH_2Cl	CH_3
(e)	CH_2I	Ph

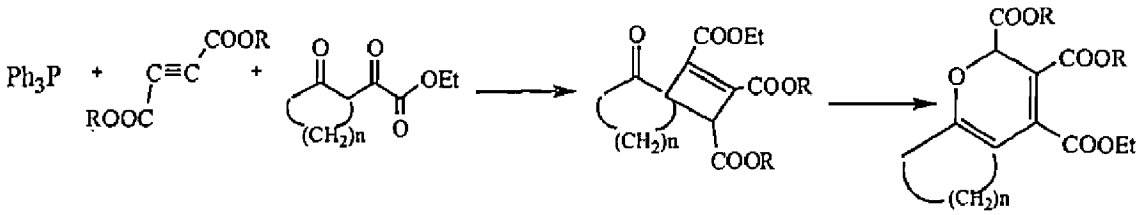
Ivanov and co-workers[5] reported the reaction of 4-hydroxy-6-methyl-pyranone and pyridinone derivatives (**20 a-c**) with Knoevenagel products (**21 a-c**) in presence of piperidine in methanol to yield substituted 2-amino-4H, 5H-pyrano [4, 3 - *b*] pyran-5-ones (**22 a-c**).



(20) (21) (22)

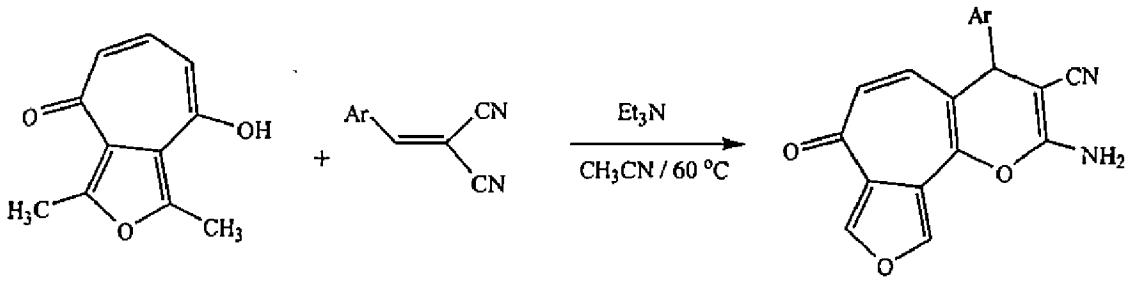
	X	Ar	Y	X	Ar	Y
(a)	O	Ph	$COOCH_3$	O	Ph	$COOCH_3$
(b)	NH	Ph	CN	NH	Ph	CN
(c)	NCH_2Ph	NO_2Ph	$COOC_2H_5$	NCH_2Ph	NO_2Ph	$COOC_2H_5$

Yavari and Bayat[6] reported that dialkylacetylenedicarboxylates (**23 a-d**) reacted smoothly with triphenylphosphine and ethyl oxo-(2-oxocycloalkyl)-ethanoates (**24 a-d**) via intramolecular Wittig reaction to produce *spiro*cyclobutene derivatives (**25 a-d**). These *spiro* systems underwent electrocyclic ring-opening reaction to produce electron-deficient 1, 3-dienes which spontaneously cyclized to 2H-pyran derivatives (**26 a-d**).



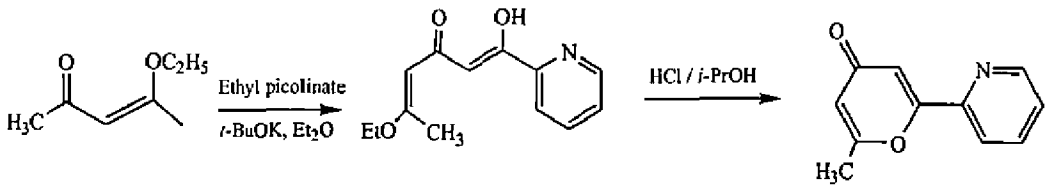
(23)	(24)	(25)		(26)	
R	**n**	**R**	**n**	**R**	**n**
(a) Me	(a) 3	(a) Me	3	(a) Me	3
(b) Et	(b) 4	(b) Et	4	(b) Et	4
(c) *i*Pr	(c) 5	(c) *i*Pr	5	(c) *i*Pr	5
(d) *i*Bu	(d) 9	(d) *i*Bu	9	(d) *i*Bu	9

Arseneva and Arsenev[7] reported that 8-hydroxy-1, 3-dimethyl-4H-cyclohepta [*c*] furan-4-one (**27**) on reaction with arylidenemalononitriles (**28 a-h**) gave the corresponding condensed 2-amino-4H-pyrans (**29 a-h**) in good yields.



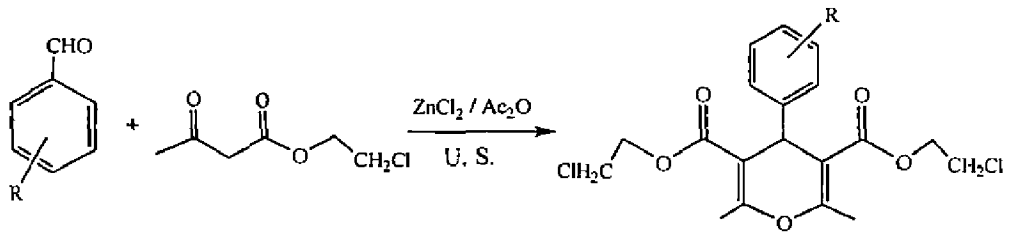
(27)	(28)	(29)
	Ar	**Ar**
	(a) Cl_2-C_6H_3	(a) Cl_2-C_6H_3
	(b) F-C_6H_4	(b) F-C_6H_4
	(c) Cl-C_6H_4	(c) Cl-C_6H_4
	(d) Br-C_6H_4	(d) Br-C_6H_4
	(e) EtO-C_6H_4	(e) EtO-C_6H_4
	(f) MeO-C_6H_4	(f) MeO-C_6H_4
	(g) MeS-C_6H_4	(g) MeS-C_6H_4
	(h) Thienyl	(h) Thienyl

Bobrov and Tyvorskii[8] reported the synthesis of 6-methyl-2-(2-pyridyl)-4H-pyran-4-one (32). The pyranone precursor (5-ethoxy-1-hydroxy-1-pyridin-2-yl hexa-1, 4-dien-3-one) (31) was prepared by Claisen condensation of acetylacetone enol ether (30) with ethyl picolinate.



(30) (31) (32)

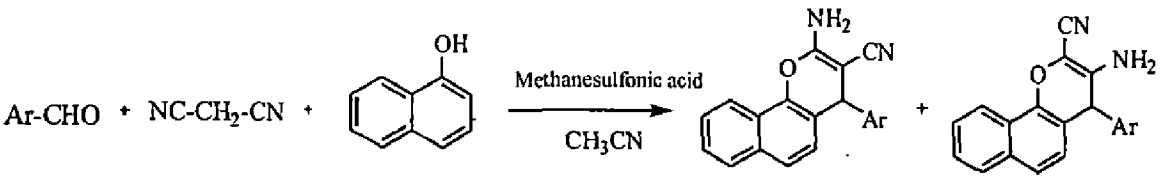
Yan and co-workers[9] reported the reaction of aromatic aldehydes (33 a-j) with 3-oxo-butyric acid-2-chloroethyl ester (34) in acetic anhydride in presence of zinc chloride, which was irradiated by an ultrasonic processor at 50 °C and 100 W to yield substituted 4-aryl-4H-pyran-3, 5-dicarboxylates (35 a-j).



(33) (34) (35)

R	R
(a) 2-F	(a) 2-F
(b) 2, 4-$(CH_3)_2$	(b) 2, 4-$(CH_3)_2$
(c) 4-Br	(c) 4-Br
(d) 4-OC_2H_5	(d) 4-OC_2H_5
(e) 2, 4-Cl, Br	(e) 2, 4-Cl, Br
(f) 2, 4-Cl, I	(f) 2, 4-Cl, I
(g) 4-F	(g) 4-F
(h) 2, 4-NO_2, CH_3	(h) 2, 4-NO_2, CH_3
(i) 2, 4-$(OH)_2$	(i) 2, 4-$(OH)_2$
(j) 3-Br	(j) 3-Br

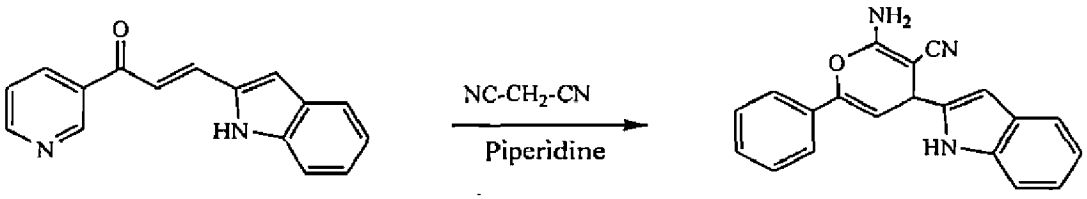
Heravi and co-workers[10] reported one-pot, three component reaction of aromatic aldehydes (36 a-e), malononitrile and α-naphthol (37) in presence of methanesulfonic acid to yield two isomers of 2-amino-4H-chromenes 38 (a-e) and 39 (a-e) in very good yields.



(36) (37) (38) (39)

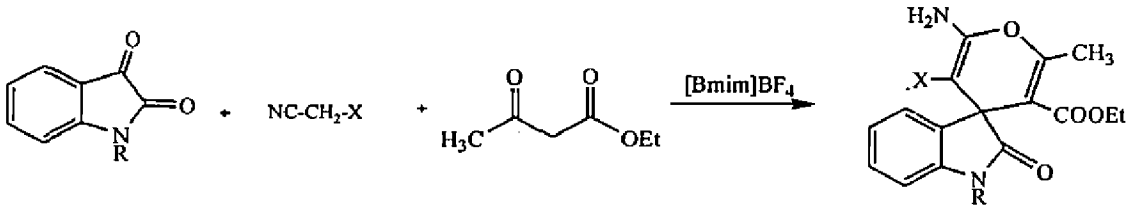
Ar	Ar	Ar
(a) $Cl_2C_6H_3$	(a) $Cl_2C_6H_3$	(a) $Cl_2C_6H_3$
(b) $(NO_2)_2C_6H_3$	(b) $(NO_2)_2C_6H_3$	(b) $(NO_2)_2C_6H_3$
(c) $(MeO)_2C_6H_3$	(c) $(MeO)_2C_6H_3$	(c) $(MeO)_2C_6H_3$
(d) $FClC_6H_3$	(d) $FClC_6H_3$	(d) $FClC_6H_3$
(e) $ClNO_2C_6H_3$	(e) $ClNO_2C_6H_3$	(e) $ClNO_2C_6H_3$

El-Latif and co-workers[11] reported the reaction of 3-β-indolylacryloylpyridine (**40**) with malononitrile in presence of piperidine to yield 2-amino-4-(3-indolyl)-6-(3-pyridyl)-pyran-3-carbonitrile (**41**).



(**40**) (**41**)

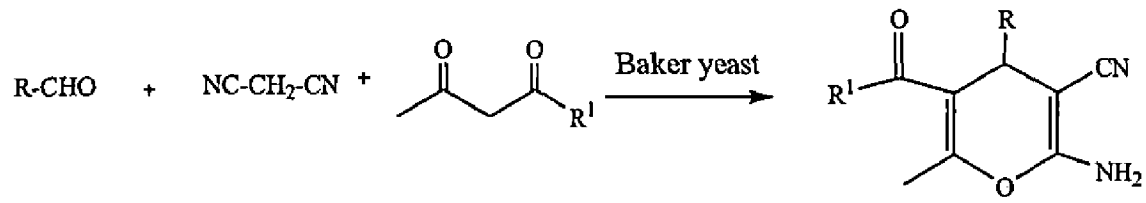
Moghadam and Miri[12] reported the reaction of isatins (**42 a-b**), malononitrile or ethyl cyanoacetate and 1, 3-dicarbonyl compound in the ionic liquid to yield *spiro* [4H-pyran-oxindole] derivatives (**43 a-d**) in better amounts.



(**42**) (**43**)

R	X	R	X
(a) H	CN	(a) H	CN
(b) Bu	COOEt	(b) H	COOEt
		(c) Bu	CN
		(d) Bu	COOEt

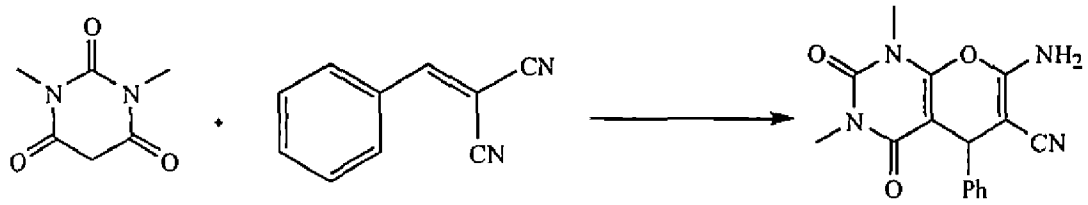
Mane and co-workers[13] reported the Baker's yeast catalyzed one-pot three-component cyclocondensation of aryl aldehydes (**44 a-g**), malononitrile and β-dicarbonyls (**45 a-c**) in dimethylacetamide solvent to obtain polyfunctionalized 4H-pyrans (**46 a-g**).



(**44**) (**45**) (**46**)

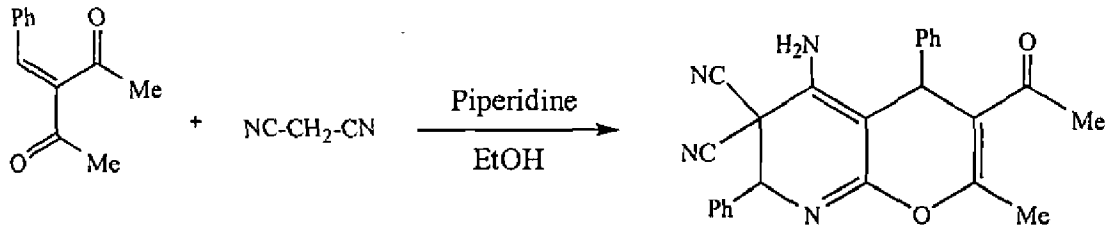
R	R^1	R	R^1
(a) $(MeO)_2C_6H_3$	(a) OEt	(a) $(MeO)_2C_6H_3$	OEt
(b) $Cl_2C_6H_3$	(b) Me	(b) $Cl_2C_6H_3$	Me
(c) $3\text{-}ClC_6H_4$	(c) OMe	(c) $3\text{-}ClC_6H_4$	OMe
(d) $4\text{-}OHC_6H_4$		(d) $4\text{-}OHC_6H_4$	OEt
(e) $4\text{-}FC_6H_4$		(e) $4\text{-}FC_6H_4$	Me
(f) 3-Pyridyl		(f) 3-Pyridyl	OMe
(g) $4\text{-}CH_3C_6H_4$		(g) $4\text{-}CH_3C_6H_4$	OEt

Seeliger and co-workers[14] have reported the formation of dihydropyrano [2, 3 - *c*] pyrimidinedione (**49**) in 80% yields by the reaction of 1, 3-dimethylbarbituric acid (**47**) with arylidene malononitrile (**48**) upon protonation.



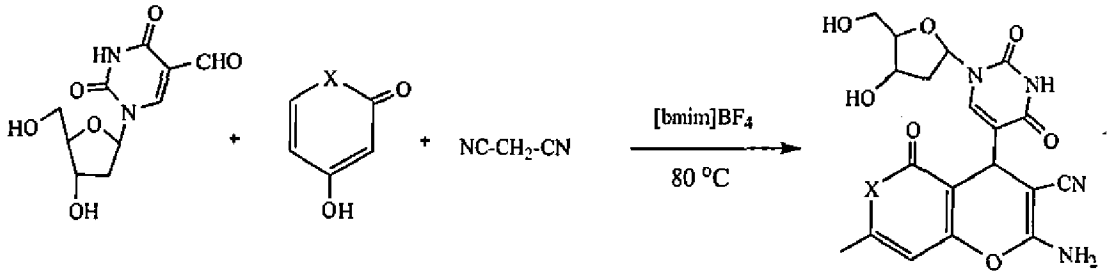
(**47**) (**48**) (**49**)

Martin and co-workers[15] reported the synthesis of pyrano [2, 3 - *b*] pyridine derivative (**51**) from malononitrile and 2-benylidene-1, 3-diketone (**50**). The compound **50** is easily accessible via Knoevenagel condensation of benzaldehyde and pentan-2, 4-dione.



(**50**) (**51**)

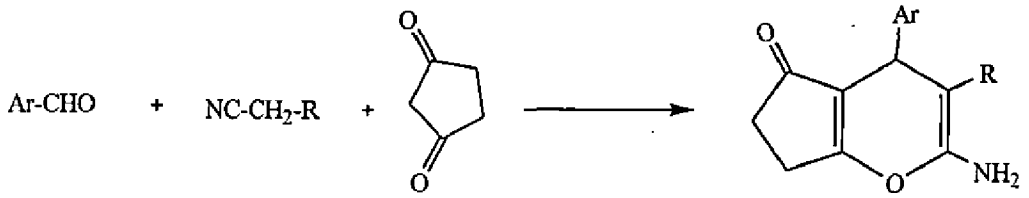
Feng and co-workers[16] reported the multi-component reactions of nucleoside (**52**), 4-hydroxy-2-pyranone (**53a**) or 4-hydroxy-pyridin-2(1H)-one (**53b**) and malononitrile in presence of ionic liquid to provide the efficient synthesis of pyrano [4, 3 - *b*] pyran nucleoside derivative (**54a**) and pyrano [3, 2 - *c*] pyridine nucleoside derivative (**54b**) which were also found as potential antiviral and anti-leishmanial agents.



(52) (53) (54)

X	X
(a) O	(a) O
(b) NH	(b) NH

Yao and co-workers[17] reported a rapid and facile synthesis of cyclopenta [*b*] pyran derivatives namely, 2-amino-4-aryl-5-oxo-tetrahydrocyclopenta [*b*] pyran-3-carbonitriles (**57 a, c**) and ethyl-2-amino-4-aryl-5-oxo-tetrahydrocyclopenta [*b*] pyran-3-carboxylates (**57 b, d**) under solvent free conditions by triturating a mixture of the three components; aromatic aldehydes (**55 a, b**), malononitrile / ethyl cyanoacetate and cyclopentadione (**56**) at 80 °C.

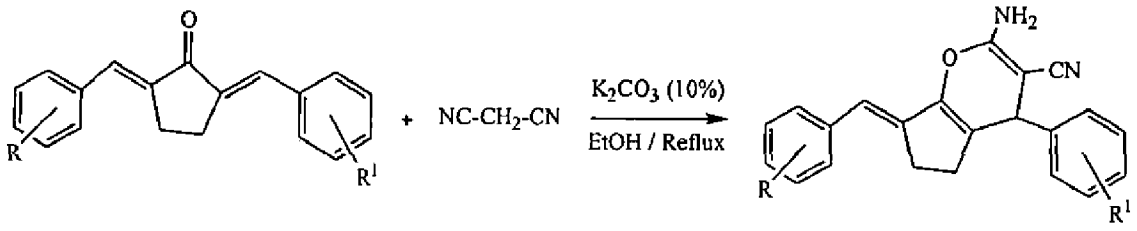


(55) (56) (57)

Ar	R
(a) C_6H_5	CN
(b) Cl-C_6H_4	COOEt

Ar	R
(a) C_6H_5	CN
(b) C_6H_5	COOEt
(c) Cl-C_6H_4	CN
(d) Cl-C_6H_4	COOEt

Karimi-Jaberi and Pooladian[18] synthesized a series of substituted 2-amino-4H-pyran-3-carbonitriles (**59 a-s**) through a one-pot condensation of malononitrile and *α, α'-bis* (arylidene) cyclopentanones (**58 a-s**) in ethanol by using K_2CO_3 as a catalyst. Short experimental reaction times, excellent yields, no need to use cumbersome apparatus for purification of the products, inexpensiveness and commercially availability of the catalyst were the advantages of this method.



(58) (59)

R	R^1
(a) CH_2	2-Cl
(b) CH_2	H
(c) CH_2	2-Cl, 4-Cl
(d) C_2H_4	2-Cl
(e) C_2H_4	H
(f) C_2H_4	2-Cl, 4-Cl
(g) C_2H_4	4-F
(h) C_2H_4	4-Br
(i) C_2H_4	4-OMe
(j) C_2H_4	4-Me
(k) C_2H_4	2-Cl, 6-F
(l) C_3H_6	H
(m) C_3H_6	2-Cl
(n) C_3H_6	2-Cl, 4-Cl
(o) C_3H_6	4-F
(p) C_3H_6	4-Br
(q) C_3H_6	4-OMe
(r) C_3H_6	4-Me
(s) C_3H_6	2-Cl, 6-F

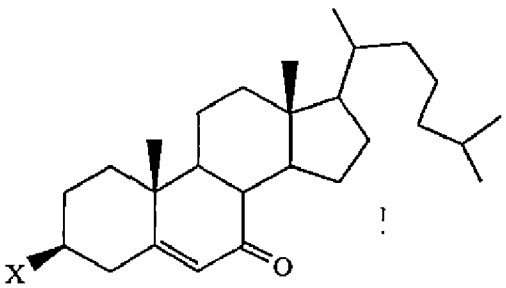
R	R^I
(a) CH_2	2-Cl
(b) CH_2	H
(c) CH_2	2-Cl, 4-Cl
(d) C_2H_4	2-Cl
(e) C_2H_4	H
(f) C_2H_4	2-Cl, 4-Cl
(g) C_2H_4	4-F
(h) C_2H_4	4-Br
(i) C_2H_4	4-OMe
(j) C_2H_4	4-Me
(k) C_2H_4	2-Cl, 6-F
(l) C_3H_6	H
(m) C_3H_6	2-Cl
(n) C_3H_6	2-Cl, 4-Cl
(o) C_3H_6	4-F
(p) C_3H_6	4-Br
(q) C_3H_6	4-OMe
(r) C_3H_6	4-OMe
(s) C_3H_6	2-Cl, 6-F

Discussion

Steroidal pyrans have always attracted considerable attention because of being an important class of biologically active molecules. Their profound physiological and clinical importance is now well validiated.[19] They have the potential to act as drugs for the treatment of a number of diseases including microbial and viral infections, tumours, diuretic disorders, and anti-leishmanial agents and antianaphylactic proximities.[16, 20-22] The diversity in the biological action might be due to the presence of different functional groups located around the tetracyclic core which serve as substrates for different targets. The advantage of employing hydrophobic steroid units is their ability to interact with cell membranes and thus pave the way for biological activity of such hybrid molecules.[23]

Attention has been devoted in the literature to the synthesis of several heterocycles that exhibit valuable pharmacological activities.[24-26] Among these are 4H-pyran derivatives which represent an important class of organic compounds with wide number of applications. They are not only used in cosmetics, pigments and biodegradable agrochemicals[27, 28] but also constitute a structural unit of many natural products.[29] These compounds have been reported to possess various pharmacological activities such as antiallergic,[28] antitumor[30] and antibacterial.[31]

The biological importance of these steroidal 4H-pyrans [28, 30, 31] and study of interesting behavior of ethyl cyanoacetate and malononitrile with simple α, β-unsaturated ketones giving 4H-pyrans encouraged us to make similar studies with steroidal α, β-unsaturated ketones. The substrates selected for synthesizing the new steroidal 4H-pyrans include cholest-5-en-7-one[32] (**60**), 3β-acetoxycholest-5-en-7-one[32] (**61**) and 3β-chlorocholest-5-en-7-one[32] (**62**). The products obtained have been characterized on the basis of spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and elemental analyses.

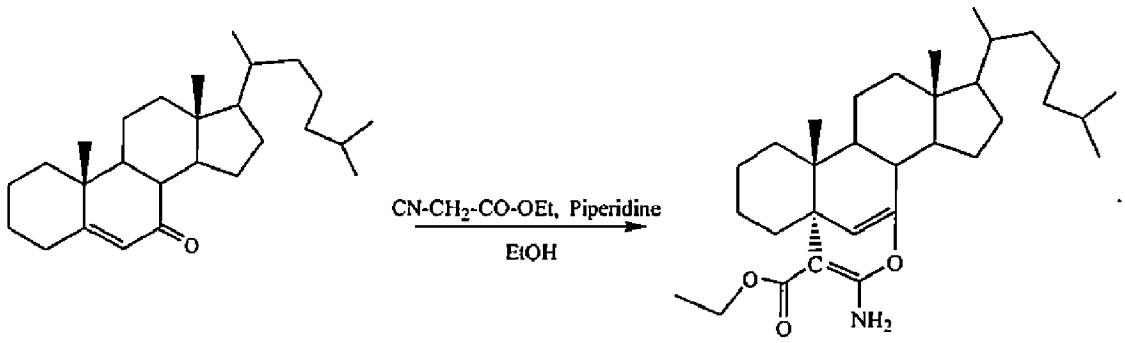


X

(**60**) H
(**61**) OAc
(**62**) Cl

Reaction of cholest-5-en-7-one (60) with ethyl cyanoacetate.

The cholest-5-en-7-one (**60**) in absolute ethanol was allowed to react with ethyl cyanoacetate in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **63**, m.p. 142 °C.



(60) **(63)**

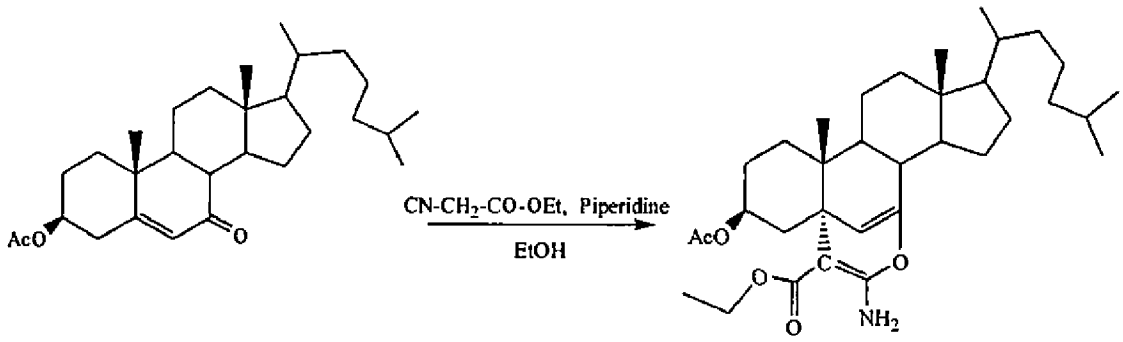
Characterization of the compound, m.p. 142 °C as 2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran (63):

The elemental analysis of compound **63** corresponded to the molecular formula $C_{32}H_{51}NO_3$. Its IR spectrum showed a band at 3290 cm^{-1} which could be assigned to NH_2 group while as the bands at 1669, 1625, 1619, 1216 and 1067 cm^{-1} were attributed to O*C*O*C*, C_6=C_7, C_2'=C_3', C_7-O and O*C*-*O* group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure **63** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 2.48 (exchangeable with D_2O) indicating the presence of N*H*$_2$ while as the singlet integrating for one proton at δ 5.3 showed the presence of C_6-olefinic proton. A quartet integrating for two protons at δ 5.7 was assigned for methylene protons (CH_3CH_2) while as a triplet integrating for three protons at δ 1.03 was assigned for methyl protons (CH_3CH_2). The prominent peaks for angular and side-chain methyl protons were observed at δ 1.13, 0.99 and 0.86. Its ^{13}C NMR spectrum displayed characteristic signals at δ 168.9 (*C*O-OEt), 166.3 (C_2'), 155.8 (C_7), 112.5 (C_6), 108.4 (C_3') and 23.6 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **63** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 497) was observed.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 2.1**), this compound can be best characterized as 2'-amino-3'-carboethoxycholest-6-eno [5, 7- *d e*] 4H-pyran (**63**).

Reaction of 3*β*-acetoxycholest-5-en-7-one (61) with ethyl cyanoacetate.

The 3*β*-acetoxycholest-5-en-7-one (**61**) in absolute ethanol was allowed to react with ethyl cyanoacetate in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **64**, m.p. 176 °C.



(61) **(64)**

Characterization of the compound, m.p. 176 °C as 3*β*-acetoxy-2'-amino-3'-carboethoxy cholest-6-eno [5, 7 - *d e*] 4H-pyran (64):

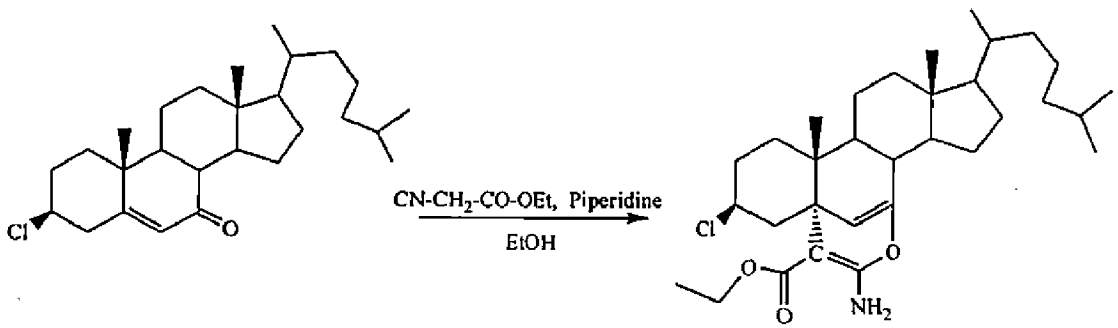
The compound **64** was correctly analyzed for the molecular formula $C_{34}H_{53}NO_5$. Its IR spectrum showed a band at 3310 cm^{-1} which could be assigned to NH_2 group. The strong absorption bands at 1740 and 1036 cm^{-1} indicated the presence of acetate group, while as the bands at 1673, 1620, 1618, 1246 and 1036 cm^{-1} were attributed to O*C*O*C*, C_6=C_7, C_2'=C_3', C_7-O and O*C*-*O* group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure **64** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 2.46 (exchangeable with D_2O) indicating the presence of N*H*$_2$ while as the singlet integrating for one proton at δ 5.3 showed the presence of C_6-olefinic proton. The quartet integrating for two protons at δ 5.7 was assigned for methylene protons (CH_3CH_2) while as the triplet integrating for three protons at δ 1.02 was assigned for CH_3CH_2. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one

proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. The three acetoxy group protons appeared at δ 2.03 as a sharp singlet. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.14, 1.02, 0.97 and 0.82. The ^{13}C NMR spectrum of compound **64** displayed characteristic signals at δ 171.2 ($OCOCH_3$), 167.9 (*C*O-OEt), 165.1 (C_2'), 157.2 (C_7), 112.5 (C_6), 104.7 (C_3') and 72.2 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **64** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 555) was found.

On the basis of above studies and its analogy with earlier compound **63**, this compound can be best characterized as 3β-acetoxy-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran (**64**).

Reaction of 3β-chlorocholest-5-en-7-one (62) with ethyl cyanoacetate.

The 3β-chlorocholest-5-en-7-one (**62**) in absolute ethanol was allowed to react with ethyl cyanoacetate in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **65**, m.p. 114 °C.



(62) **(65)**

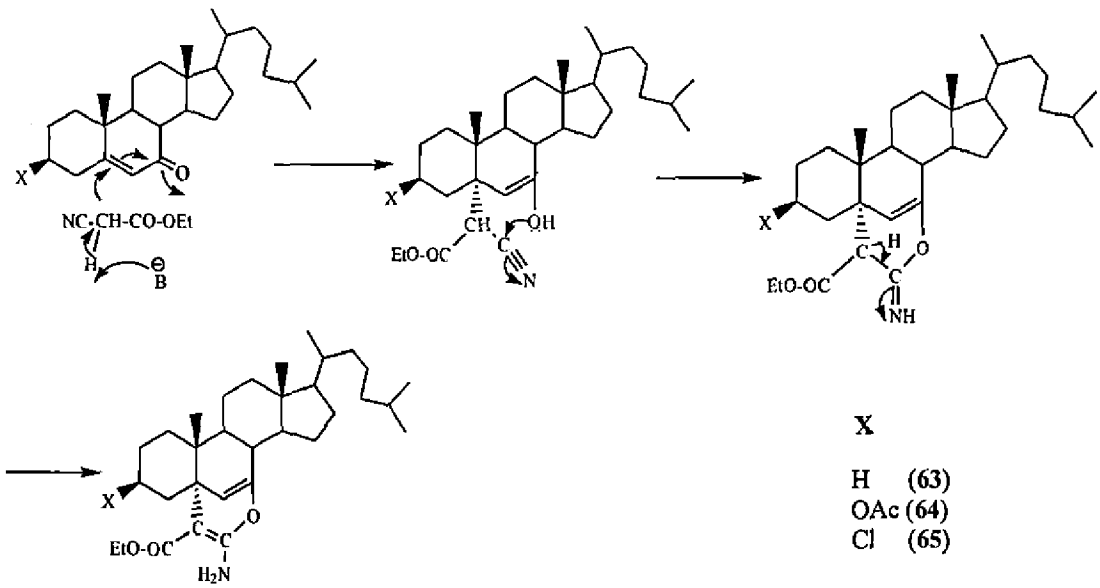
Characterization of the compound, m.p. 114 °C as 3β-chloro-2'-amino-3'-carboethoxy cholest-6-eno [5, 7 - *d e*] 4H-pyran (65):

The compound **65** was correctly analyzed for the molecular formula $C_{32}H_{50}ClNO_3$ (Beilstein positive). Its IR spectrum showed a band at 3296 cm^{-1} which could be assigned to NH_2 group while as the bands at 1661, 1622, 1618, 1297, 1080 and 749 cm^{-1} were attributed to O*C*O*C*, C_6=C_7, C_2'=C_3', C_7-O, O*C*-*O* and C-Cl group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure **65** was well supported by its ^1H NMR spectrum which displayed a broad singlet integrating for two

protons at δ 2.7 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for one proton at δ 5.6 showed the presence of C_6-olefinic proton. The quartet integrating for two protons at δ 6.07 was assigned for CH_3CH_2 while as the triplet integrating for three protons at δ 1.01 was assigned for CH_3CH_2. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.13, 1.01, 0.96 and 0.82. The ^{13}C NMR spectrum of compound **65** displayed characteristic signals at δ 166.4 (*C*O-OEt), 163.1 (C_2'), 156.4 (C_7), 111.6 (C_6), 106.5 (C_3') and 50.7 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **65** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 529/531) was found.

The above data led to the structure of compound **65** as, 3β-chloro-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran.

Formation of steroidal 4H-pyrans (**63-65**) under the condition case and in the light of available literature[11, 18] may be shown according to the proposed mechanism (**Scheme 2.1**).



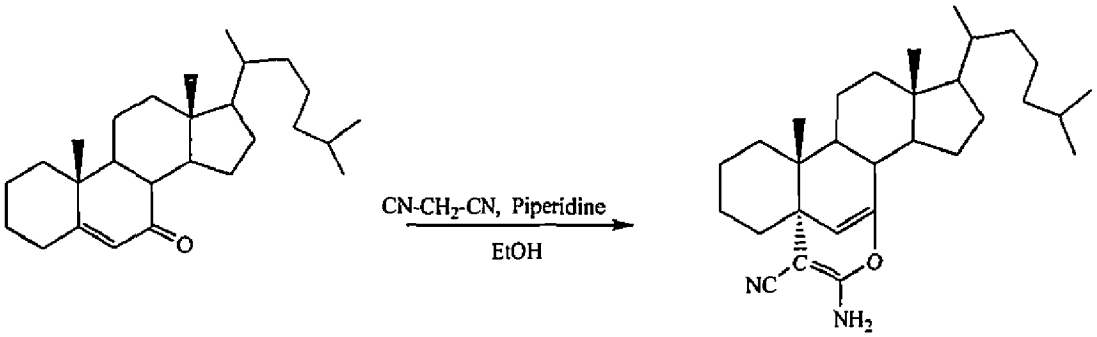
Scheme 2.1 Mechanism for the formation of 2'-amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran derivatives (**63-65**)

Work published;

Synthesis, molecular docking and biological evaluation of new steroidal 4H-pyrans, Shamsuzzaman, **Ayaz Mahmood Dar**, et al, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 117 (2014) 493-501

Reaction of cholest-5-en-7-one (60) with malononitrile.

The cholest-5-en-7-one (60) in absolute ethanol was allowed to react with malononitrile in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **66**, m.p. 149 °C.



(60) **(66)**

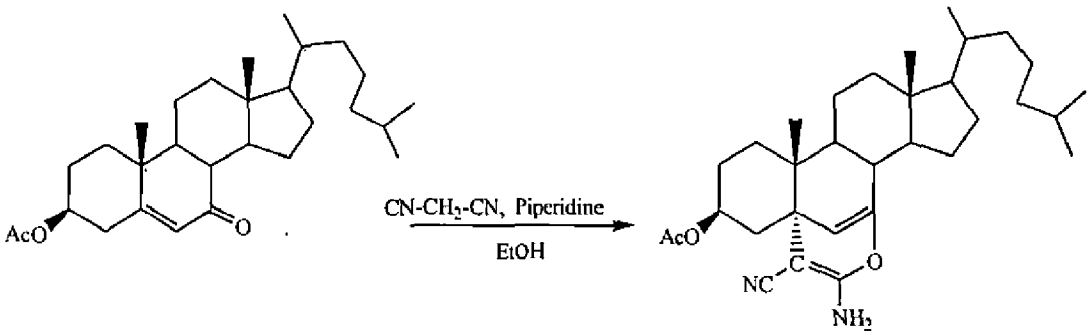
Characterization of the compound, m.p. 149 °C as 2'-amino-3'-cyanocholest-6-eno [5, 7 - *d e*] 4H-pyran (66):

The elemental analysis of compound **66** corresponded to the molecular formula $C_{30}H_{46}N_2O$. Its IR spectrum showed a band at 3363 cm^{-1} which could be assigned to NH_2 group while as the bands at 2234, 1630, 1617, 1078 and 1037 cm^{-1} were attributed to C≡N, C_6=C_7, C_2'=C_3', C_7-O and C-N group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure **66** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 2.67 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for one proton at δ 5.26 showed the presence of C_6-olefinic proton. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.17, 1.04, 1.02 and 0.86. The ^{13}C NMR spectrum of compound **66** displayed characteristic signals at δ 164 (C_2'), 154.3 (C_7), 134.4 (*C*≡N), 113.3 (C_6), 66.7 (C_3') and 23.2 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **66** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 450) was found.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 2.2**), this compound can be best characterized as 2'-amino-3'-cyanocholest-6-eno [5, 7 - *d e*] 4H-pyran (**66**).

Reaction of 3*β*-acetoxycholest-5-en-7-one (61) with malononitrile.

The 3*β*-acetoxycholest-5-en-7-one (**61**) in absolute ethanol was allowed to react with malononitrile in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **67**, m.p. 165 °C.



(61) **(67)**

Characterization of the compound, m.p. 165 °C as 3*β*-acetoxy-2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran (67):

The compound **67** was correctly analyzed for the molecular formula $C_{32}H_{48}N_2O_3$. Its IR spectrum showed a band at 3340 cm^{-1} which could be assigned to NH_2 group. The IR spectrum of the compound **67** also exhibited strong absorption bands at 1713 and 1036 cm^{-1} indicating the presence of acetate group and the bands at 2203, 1625, 1620, 1065 and 1019 cm^{-1} were attributed to C≡N, C_6=C_7, C_2'=C_3', C_7-O and C-N group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure **67** was well supported by its ^{1}H NMR spectrum which displayed broad singlet integrating for two protons at δ 2.5 (exchangeable with D_2O) indicating the presence of N*H*$_2$ while as the singlet integrating for one proton at δ 5.69 showed the presence of C_6-olefinic proton. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. The acetoxy group protons appeared at δ 2.05 as a sharp singlet. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.14, 1.04,

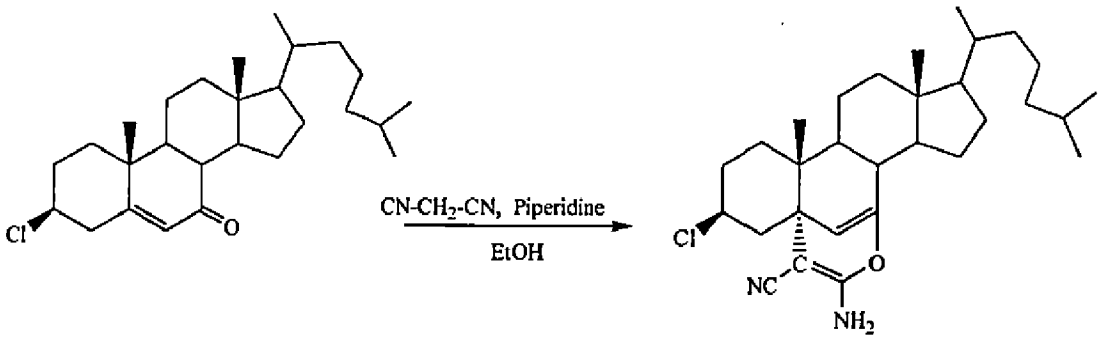
THESIS

1.02 and 0.82. The ^{13}C NMR spectrum of compound **67** displayed characteristic signals at δ 173.1 ($OCOCH_3$), 168 (C_2'), 157.2 (C_7), 132.2 ($C\equiv N$), 111.6 (C_6), 72.2 (C_3) and 67.2 (C_3'). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **67** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 508) was found.

On the basis of above studies and its analogy with earlier compound **66**, this compound can be best characterized as 3*β*-acetoxy-2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran (**67**).

Reaction of 3*β*-chlorocholest-5-en-7-one (62) with malononitrile.

The 3*β*-chlorocholest-5-en-7-one (**62**) in absolute ethanol was allowed to react with malononitrile in presence of piperidine. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **68**, m.p. 139 °C.



(62) **(68)**

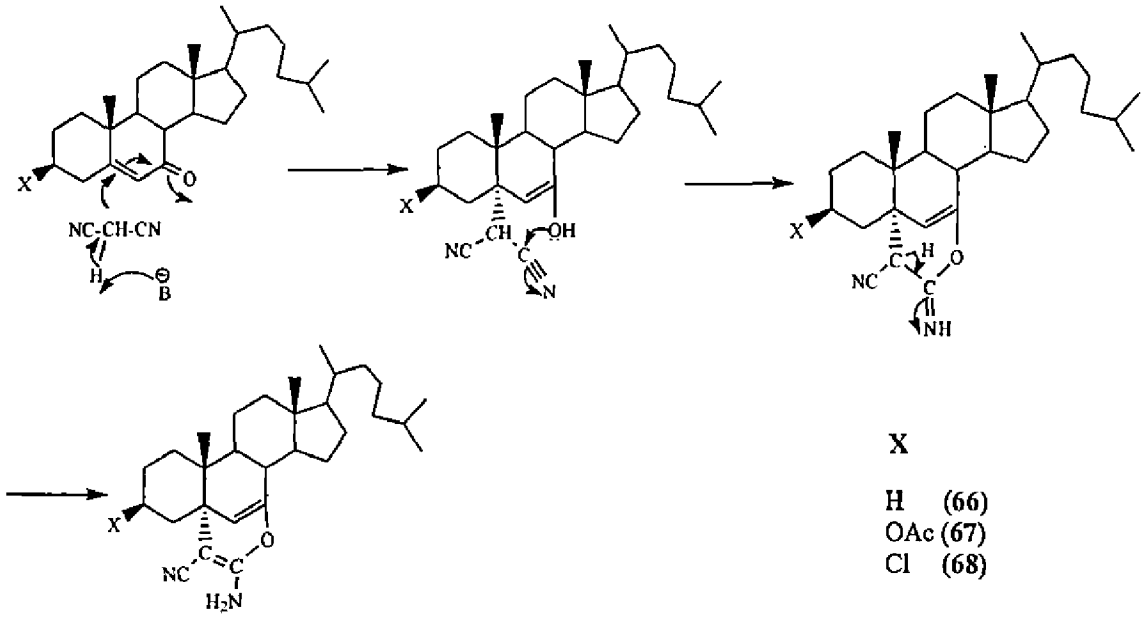
Characterization of the compound, m.p. 139 °C as 3*β*-chloro-2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran (68):

The compound **68** was correctly analyzed for the molecular formula $C_{30}H_{45}ClN_2O$ (Beilstein positive). Its IR spectrum showed a band at 3396 cm^{-1} which could be assigned to NH_2 group while as the bands at 2259, 1630, 1625, 1116, 1054 and 742 cm^{-1} were attributed to $C\equiv N$, $C_6=C_7$, $C_2'=C_3'$, C_7-O, C-N and C-Cl group, respectively. These values supported the presence of 4H-pyran moiety[33] in the product molecule. The structure of **68** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons

at δ 2.72 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for one proton at δ 5.3 showed the presence of C_6-olefinic proton. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.16, 1.04, 1.02 and 0.82. The ^{13}C NMR spectrum of compound **68** displayed characteristic signals at δ 166 (C_2'), 155.2 (C_7), 129.2 (C=N), 114.6 (C_6), 67.3 (C_3') and 50.2 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **68** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 484/486) was found.

The above data led to the structure of compound **68** as, 3β-chloro-2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran.

Formation of steroidal 4H-pyrans (**66-68**) under the condition case and in the light of available literature[11, 18] may be shown according to the proposed mechanism (**Scheme 2.2**).



Scheme 2.2 Mechanism for the formation of 2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran derivatives (**66-68**)

Work published;

Synthesis and biological studies of steroidal pyran based derivatives, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Photochemistry and Photobiology B: Biology* 129 (2013) 36-47

Experimental

All the melting points were determined in degrees Celsius on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Perkin Elmer RXI Spectrophotometer and values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of boiling point 60-80 °C. Sodium sulfate (anhydrous) was used as a drying agent.

Cholest-5-ene:

3β-Chlorocholest-5-ene (10 g) was dissolved in warm amyl alcohol (230 mL) and sodium metal (20 g) was added in small portions to the solution with continuous stirring over the period of 8 h. The reaction mixture was warmed occasionally. When all the sodium metal was dissolved, the reaction mixture was poured into water, acidified with dilute hydrochloric acid and allowed to stand overnight. A white crystalline solid thus obtained was filtered under suction, washed thoroughly with water and air dried. Recrystallization of the crude material from acetone gave cholest-5-ene (8.3 g) in cubes, m.p. 92 °C (reported m.p. 89-91 °C).[32]

Cholest-5-en-7-one (60):

A solution of *tert*-butyl chromate [*tert*-butyl alcohol (60 mL), chromium trioxide (20 g), acetic acid (84 mL) and acetic anhydride (10 mL)] were added at 0 °C to a solution of cholest-5-ene (8 g) in CCl_4 (150 mL), glacial acetic acid (30 mL) and acetic anhydride (10 mL). The contents were refluxed for 3 h and then diluted with water. The organic layer was washed with sodium bicarbonate solution (5 %) and water and dried over anhydrous sodium sulfate. Evaporation of solvents under reduced pressure provided an oil which was crystallized from methanol to give cholest-5-en-7-one (3.1 g) as needles, m.p. 128 °C (reported m. p. 125-129 °C).[32]

3β-Acetoxycholest-5-ene:

A mixture of cholesterol (50 g), pyridine (75 mL freshly distilled over KOH) and freshly distilled acetic anhydride (50 mL) was heated on a water bath for 2 h. The resulting brown colored reaction mixture was poured into crushed ice-water mixture with stirring. A light brown solid thus obtained was filtered under suction, washed with water until free from

pyridine and air dried. The crude product on crystallization from acetone gave pure 3β-acetoxycholest-5-ene (45 g), m.p. 115 °C (reported m.p. 115-116 °C).[34]

3β-Acetoxycholest-5-en-7-one (61):

To a solution of 3β-acetoxycholest-5-ene (8 g) in carbon tetrachloride (150 mL), acetic acid (30 mL) and acetic anhydride (10 mL) was added at 0 °C a solution of *tert*-butyl chromate [*tert*-butyl alcohol (60 mL), chromium trioxide (20 g), acetic acid (84 mL) and acetic anhydride (10 mL)]. The reaction mixture was heated under reflux for 4 h and then it was diluted with cold water. The organic layer was taken in diethyl ether, washed with water, sodium bicarbonate solution (5%) and again with water and dried over anhydrous sodium sulfate. Evaporation of solvents under reduced pressure provided an oil which was crystallized from methanol to give 3β-acetoxycholest-5-en-7-one (**61**) as crystals (3.5 g), m.p. 156 °C (reported m. p. 156-158 °C).[32]

3β-Chlorocholest-5-ene:

Freshly purified thionyl chloride (40 mL) was added gradually to cholesterol (50 g) at room temperature. A vigorous reaction ensured with evolution of gaseous products. When the reaction slackened, the mixture was gently heated at temperature 50-60 °C on water bath for 1 h and then poured into crushed ice-water with stirring. The yellow solid thus obtained was filtered under suction and washed several times with ice-cold water and air dried. Recrystallization of crude product from acetone gave 3β-chlorocholest-5-ene (42 g), m.p. 95-96 °C (reported m.p. 96-97 °C).[35] It gave positive Beilstein test and a yellow color with tetranitromethane in chloroform.

3β-Chlorocholest-5-en-7-one (62):

A solution of *tert*-butyl chromate [*tert*-butyl alcohol (60 mL), chromium trioxide (20 g), acetic acid (84 mL) and acetic anhydride (10 mL)] were added at 0 °C to a solution of 3β-chlorocholest-5-ene (8 g) in CCl_4 (150 mL), glacial acetic acid (30 mL) and acetic anhydride (10 mL). The contents were refluxed for 3 h and then diluted with water. The organic layer was washed with sodium bicarbonate solution (5 %) and water and dried over anhydrous Na_2SO_4. Evaporation of solvents under reduced pressure furnished the product as an oil which was crystallized from methanol to give 3β-chlorocholest-5-en-7-one (**62**) (3.3 g) as needles, m.p. 144 °C (reported m. p. 144-145 °C). [32]

Reaction of cholest-5-en-7-one derivatives (60-62) with ethyl cyanoacetate/malononitrile:

To a solution of cholest-5-en-7-one derivatives (**60-62**) (1 mmol) in absolute ethanol (20 mL) was added ethyl cyanoacetate/ malononitrile in equimolar ratio followed by piperidine (1.5 mL). The reaction mixture was refluxed for 11 h. The progress of reaction was monitored by TLC. After completion of the reaction, excess solvent was reduced to three fourths of the original volume under reduced pressure. The reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Evaporation of solvents and crystallization of the oily residue from methanol afforded corresponding products (**63-68**).

2'-Amino-3'-carboethoxycholest-6-eno [5, 7- d e] 4H-pyran (63):

Yield 70%; m.p. 142 °C; Analysis found: C 77.27, H 10.26, N 2.81%. $C_{32}H_{51}NO_3$ requires: C 77.18, H 10.06, N 2.77%; IR (KBr): ν_{max} 3290 (NH_2), 1669 (O*C*O*C*), 1625 (C_6=C_7), 1619 (C_2'=C_3'), 1216, 1067 (C-O); ^{1}H NMR ($CDCl_3$): δ 5.7 (2H, q, C*H*$_2$), 5.3 (1H, s, C_6 *H*), 2.48 (2H, s, N*H*$_2$, exchangeable with D_2O), 1.13 (3H, s, C_{10}-C*H*$_3$), 1.2 (3H, s, C_{13}-C*H*$_3$), 1.03 (3H, t, C*H*$_3$), 0.99 and 0.86 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 168.9 (*C*O-OEt), 166.3 (C_2'), 155.8 (C_7), 112.5 (C_6), 108.4 (C_3'), 23.6 (C_3); MS: *m/z* 497 [$M^{+\cdot}$].

3β-Acetoxy-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - d e] 4H-pyran (64):

Yield 70%; m.p. 176 °C; Analysis found: C 73.51, H 9.54, N 2.52%. $C_{34}H_{53}NO_5$ requires: C 73.44, H 9.37, N 2.48%; IR (KBr): ν_{max} 3310 (NH_2), 1740 (O*C*O*C*H$_3$), 1673 (O*C*O*C*), 1620 (C_6=C_7), 1618 (C_2'=C_3'), 1246, 1036 (C-O); ^{1}H NMR ($CDCl_3$): δ 5.7 (2H, q, C*H*$_2$), 5.3 (1H, s, C_6 *H*), 4.7 (1H, m, $C_3\alpha$-*H*, *W*½ = 15 Hz), 2.46 (2H, s, N*H*$_2$, exchangeable with D_2O), 2.03 (3H, s, OCOC*H*$_3$), 1.20 (3H, s, C_{13}-C*H*$_3$), 1.14 (3H, s, C_{10}-C*H*$_3$), 1.02 (3H, t, C*H*$_3$), 0.97 and 0.82 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 171.2 (O*C*OCH$_3$), 167.9 (*C*O-OEt), 165.1 (C_2'), 157.2 (C_7), 112.5 (C_6), 104.7 (C_3'), 72.2 (C_3); MS: *m/z* 555 [$M^{+\cdot}$].

3β-Chloro-2'-amino-3'-carboethoxycholest-6-eno [5, 7 - d e] 4H-pyran (65):

Yield 70%; m.p. 114 °C; Analysis found: C 72.58, H 9.48, N 2.64%. $C_{32}H_{50}ClNO_3$ requires: C 72.45, H 9.34, N 2.62%; IR (KBr): ν_{max} 3296 (NH_2), 1661 (O*C*O*C*), 1622 (C_6=C_7), 1618 (C_2'=C_3'), 1297, 1080 (C-O), 749 (C_3-Cl); ^{1}H NMR ($CDCl_3$): δ 6.07 (2H, q, C*H*$_2$), 5.6 (1H, s, C_6 *H*), 3.9 (1H, m, $C_3\alpha$-*H*, *W* ½ = 17 Hz), 2.7 (2H, s, N*H*$_2$, exchangeable with D_2O), 1.2 (3H, s, C_{13}-C*H*$_3$), 1.13 (3H, s, C_{10}-C*H*$_3$), 1.01 (3H, t, C*H*$_3$), 0.96 and 0.82 (other methyl protons);

^{13}C NMR ($CDCl_3$): δ 166.4 (*C*O-OEt), 163.1 (C_2'), 156.4 (C_7), 111.6 (C_6), 106.5 (C_3'), 50.7 (C_3); MS: *m/z* 529/531 [M^+].

2'-Amino-3'-cyanocholest-6-eno [5, 7- d e] 4H-pyran (66):

Yield 75%; m.p. 149 °C; Analysis found: C 80.0, H 10.22, N 6.22%. $C_{30}H_{46}N_2O$ requires: C 79.96, H 10.12, N 6.17%; IR (KBr): ν_{max} 3363 (NH_2), 2234 (CN), 1630 (C_6=C_7), 1617 (C_2'=C_3'), 1078 (C-O), 1037 (C-N); ^1H NMR ($CDCl_3$): δ 5.26 (1H, s, C_6H), 2.67 (2H, s, NH_2, exchangeable with D_2O), 1.2 (3H, s, C_{13}-CH_3), 1.17 (3H, s, C_{10}-CH_3), 1.04 and 1.02 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 164 (C_2'), 154.3 (C_7), 134.4 (*C*≡N), 113.3 (C_6), 66.7 (C_3'), 23.2 (C_3); MS: *m/z* 450 [M^+].

3β-Acetoxy-2'-amino-3'-cyanocholest-6-eno [5, 7 - d e] 4H-pyran (67):

Yield 75%; m.p. 165 °C; Analysis found: C, 75.59, H 9.44, N 5.51%. $C_{32}H_{48}N_2O_3$ requires: C 75.43, H 9.26, N 5.44%; IR (KBr): ν_{max} 3340 (NH_2), 2203 (CN), 1713 ($OCOCH_3$), 1625 (C_6=C_7), 1620 (C_2'=C_3'), 1065 (C-O), 1019 (C-N); ^1H NMR ($CDCl_3$): δ 5.69 (1H, s, C_6H), 4.7 (1H, m, $C_3\alpha$-*H*, *W½* = 15 Hz), 2.5 (2H, s, NH_2, exchangeable with D_2O), 2.05 (3H, s, $OCOCH_3$), 1.2 (3H, s, C_{13}-CH_3), 1.14 (3H, s, C_{10}-CH_3), 1.04 and 1.02 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 173.1 ($OCOCH_3$), 168 (C_2'), 157.2 (C_7), 132.2 (*C*≡N), 111.6 (C_6), 72.2 (C_3), 67.2 (C_3'); MS: *m/z* 508 [M^+].

3β-Chloro-2'-amino-3'-cyanocholest-6-eno [5, 7 - d e] 4H-pyran (68):

Yield 75%; m.p. 139 °C; Analysis found: 74.38, H 9.29, N 5.78%. $C_{30}H_{45}ClN_2O$ requires: C 74.26, H 9.12, N 5.61%; IR (KBr): ν_{max} 3396 (NH_2), 2259 (CN), 1630 (C_6=C_7), 1625 (C_2'=C_3'), 1116 (C-O), 1054 (C-N), 742 (C-Cl); ^1H NMR ($CDCl_3$): δ 5.3 (1H, s, C_6H), 3.9 (1H, m, $C_3\alpha$-*H*, *W½* = 17 Hz), 2.72 (2H, s, NH_2, exchangeable with D_2O), 1.23 (3H, s, C_{13}-CH_3), 1.16 (3H, s, C_{10}-CH_3), 1.04 and 1.02 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 166 (C_2'), 155.2 (C_7), 129.2 (*C*≡N), 114.6 (C_6), 67.3 (C_3'), 50.2 (C_3); MS: *m/z* 484/486 [M^+].

References

References

1. S. Masamune, N. T. Castellucci, *J. Am. Chem. Soc.*, **84**, 2452 (1962)
2. L. C. Dorman, *J. Org. Chem.*, **32**, 4105 (1967)
3. N. S. Babu, N. Pasha, K. T. V. Rao, P. S. S. Prasad, N. Lingaiah, *Tetrahedron Lett.*, **49**, 2730 (2008)
4. V. I. Tyvorskii, D. N. Bobrov, O. G Kulinkovich, *Tetrahedron*, **54**, 2819 (1998)
5. E. V. Stoyanov, I. C. Ivanov, D. Heber, *Molecules*, **5**, 19 (2000)
6. I. Yavari, M. Bayat, *Tetrahedron*, **59**, 2001 (2003)
7. M. Yu. Arsen'eva, V. G. Arsen'ev, *Chem. Heterocycl. Comp.*, **44**, 136 (2008)
8. D. N. Bobrov, V. I. Tyvorskii, *Tetrahedron*, **66**, 5432 (2010)
9. C. Ni, X. Song, H. Yan, X. Song, R. Zhong, *Ultrason. Sonochem.*, **17**, 367 (2010)
10. M. M. Heravi, B. Baghernejad, H. A. Oskooie, *J. Chinese Chem. Soc.*, **55**, 659 (2008)
11. N. A. A. El-Latif, A. E. E. Amr, A. A. Ibrahiem, *Monatsh. Chem.*, **138**, 559 (2007)
12. K. R. Moghadam, L. Y. Miri, *Tetrahedron*, **67**, 5693 (2011)
13. U. R. Pratap, D. V. Jawale, P. D. Netankar, R. A. Mane, *Tetrahedron Lett.*, **52**, 581 (2011)
14. F. Seeliger, S. T. A. Berger, G. Y. Remennikov, K. Polborn, H. Mayr, *J. Org. Chem.*, **72**, 9170 (2007)
15. N. Martin, C. Seoane, J. L. Soto, *Tetrahedron*, **44**, 5861 (1988).
16. X. F. D. Feng, Y. Qua, X. Zhang, J. Wang, P. M. Loiseau, G. Andrei, R. Snoeck E. D. Clercq, *Bioorg. Med. Chem. Lett.*, **20**, 809 (2010)
17. C. Yao, B. Jiang, T. Li, B. Qin, X. Feng, H. Zhang, C. Wang, S. Tu, *Bioorg. Med. Chem. Lett.*, **21**, 599 (2011)
18. Z. K. Jaberi, B. Pooladian, *The Scientific World Journal*, Article ID 208796, (2012) doi:10.1100/2012/208796
19. S. C. Kuo, L. J. Huang, H. Nakamura, *J. Med. Chem.* **27** 539 (1984)
20. R. W. De Simone, K. S. Currie, S. A. Mitchell, J. W. Darrow, D. A. Pippin, *Comb. Chem. High Throughput Screen*, **7**, 473 (2004)
21. J. Safari, Z. Zarnegar, M. Heydarian, *Bull. Chem. Soc. Jpn.* **85**, 1332 (2012)
22. M. M. Heravi, B. Baghernejad, H. A. Oskooie, *J. Chinese Chem. Soc.* **55**, 659 (2008)
23. D. B. Gower, H. L. J. Makin, *Biochemistry of steroid hormones*. Oxford/ London/ Edinburgh, Blackwell Scientific Publications, p. 117, 1984
24. M. Watanabe, S. Mataka, T. Thiemann, *Steroids*, **70**, 856 (2005)
25. J. Wolfling, L. Hackler, E. Mernyak, G. Schneider, I. Toth, M. Szecsi, J. Julesz, P.

Aligarh Muslim University, Aligarh

Sohar, A. Csampai, *Steroids*, **6**, 451 (2004)

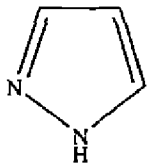
26. E. Frank, B. Kazi, K. Ludanyi, G. Keglevich. *Tetrahedron Lett.*, **47**, 1105 (2006)
27. J. C. Hackett, Y. W. Kim, B. Su, R. W. Brueggemeier, *Bioorg. Med. Chem.*, **13**, 4063 (2005)
28. E. C. Witte, P. Neubert, A. Roesch, Ger. Offen. DE 3427985, *Chem. Abst.*, **104**, 224915f (1986)
29. S. Hatakeyama, N. Ochi, H. Numata, S. Takano, *Chem. Commun.*, **17**, 1202 (1988)
30. J. L. Wang, D. Liu, Z. J. Zhang, S. Shan, X. Han, S. M. Srinivasula, C. M. Croce, E. S. Alnemri, Z. Huang, *PNAS*, **97**, 7124 (2000)
31. I. J. S. Fairlamb, L. R. Marrison, J. M. Dickinson, F. J. Lu, J. P. Schmidt, *Bioorg. Med. Chem.*, **12**, 4285 (2004)
32. W. G. Dauben, K. H. Takemura, *J. Am. Chem. Soc.*, **75**, 6302 (1953)
33. G. Socrates, *Infrared Charatcteristic Group Frequencies* (John-wiley & Sons) p. 102, 1980
34. L. F. Fieser, M. Fieser, "*Steroids*" Reinhold Publishing Corporation, New York, p. 28, 1959
35. C. W. Shoppe, R. H. Jenkins, G. H. R. Summers, *J. Chem. Soc.*, 1657 (1958)

Chapter-3

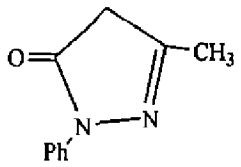
Synthesis of steroidal pyrazoles and pyrazolones

Theoretical

The pyrazole system (1) consists of a doubly unsaturated five membered ring with two adjacent nitrogen atoms. Knorr,[1, 2] first synthesized compounds containing this system in 1883 by the reaction of ethyl acetoacetate with phenyl hydrazine, which yielded 1-phenyl-3-methyl-5-pyrazolone (2). Knorr[3] introduced the name pyrazole for these compounds to denote that the nucleus was derived from pyrrole by the replacement of a carbon by nitrogen. They synthesized many members of this class and systematically investigated their properties.

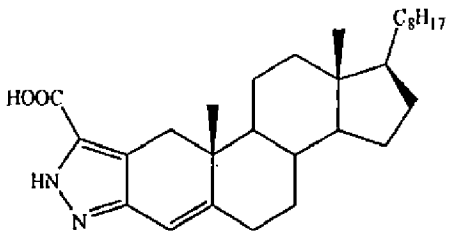


(1)



(2)

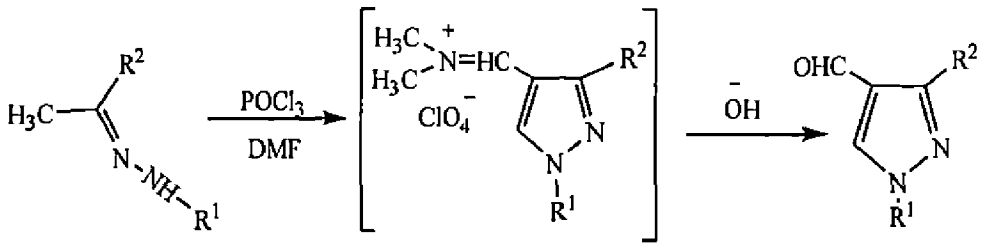
Literature survey has revealed that till 1930s very little had been done for the synthesis of steroidal pyrazole derivatives. Probably the first steroidal pyrazole was reported in 1938 by Ruzicka *et al.*[4] and only a single derivative; cholest-4-eno [3, 2 - *c*] pyrazole-5-carboxylic acid (3) was mentioned.



(3)

After a considerable span of time, much attention has been paid by a number of organic chemists towards the synthesis of several steroidal pyrazoles. The effect on different biological activities produced by the fusion of a pyrazole ring to the steroid nucleus has prompted us to investigate such type of compounds. The compounds containing pyrazole ring system can be synthesized by different routes and here we have summarized only important examples, as below.

Kira *et al.*[5] reported that treatment of acetophenone phenylhydrazone (**4 a-d**) with two moles of DMF-$POCl_3$ in DMF at 70-80 °C for 6 h gave immonium perchlorate. Alkaline hydrolysis of immonium perchlorate afforded 1, 3-diarylpyrazole-4-carboxaldehyde (**5 a-d**).

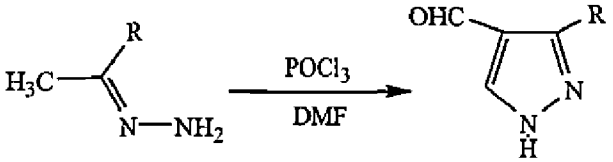


(4) (5)

R^1	R^2
(a) C_6H_5	C_6H_5
(b) C_6H_5	CH_3
(c) C_6H_5	p-$C_6H_4NO_2$
(d) p-$C_6H_4NO_2$	C_6H_5

R^1	R^2
(a) C_6H_5	C_6H_5
(b) C_6H_5	CH_3
(c) C_6H_5	p-$C_6H_4NO_2$
(d) p-$C_6H_4NO_2$	C_6H_5

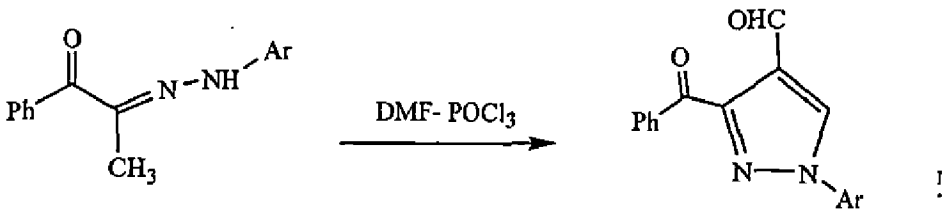
Kira *et al.*[6] synthesized 3-substituted pyrazole-4-carboxaldehyde (**7 a-f**) by the reaction of semicarbazones (**6 a-f**) with two moles of DMF-$POCl_3$ in DMF.



(6) (7)

R	R
(a) C_6H_5	(a) C_6H_5
(b) o-CH_3O-C_6H_4	(b) o-CH_3O-C_6H_4
(c) p-NO_2-C_6H_4	(c) p-NO_2-C_6H_4
(d) m-NO_2-C_6H_4	(d) m-NO_2-C_6H_4
(e) 2-thienyl	(e) 2-thienyl
(f) CH_3	(f) CH_3

Bavatenko and co-workers[7] reported substituted pyrazoles (**9 a-d**) by cyclizing aryl hydrazones (**8 a-d**) under Vilsmeier conditions.



(8) (9)

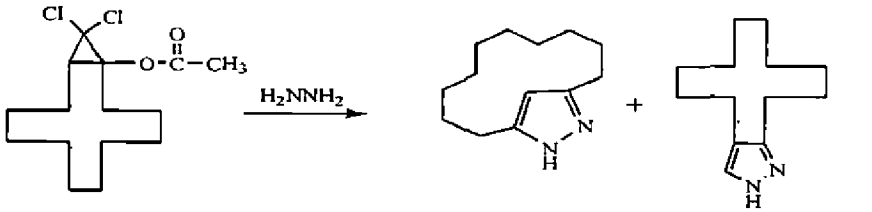
Ar	Ar
(a) C_6H_5	(a) C_6H_5
(b) 4-F-C_6H_4	(b) 4-F-C_6H_4
(c) 4-CH_3-C_6H_4	(c) 4-CH_3-C_6H_4
(d) 4-CH_3O-C_6H_4	(d) 4-CH_3O-C_6H_4

3, 5-Disubstituted pyrazoles (**11 a-d**) were obtained by the reaction of *gem*-dichlorocyclopropylacetates (**10 a, b**) with 4 equivalents of hydrazine or phenyl hydrazine at room temperature while metacyclophane (**13a**) and its isomer (**13b**) were obtained from 1-acetoxy-2, 2-dichlorobicyclo [10.1.0] tridecane (**12**) with hydrazine under same condition.[8]

(10) (11)

	R^1	R^2
(a)	CH_3	H
(b)	C_6H_5	C_6H_5

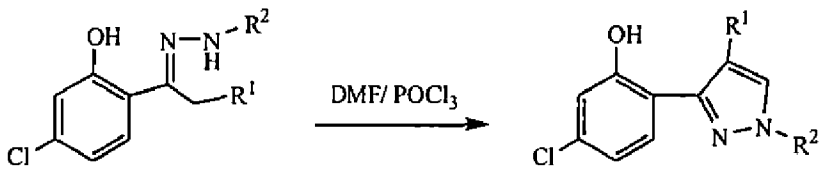
	R^1	R^2	R^3
(a)	CH_3	H	H
(b)	C_6H_5	C_6H_5	H
(c)	CH_3	H	C_6H_5
(d)	C_6H_5	C_6H_5	C_6H_5



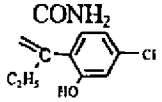
(12) (13a) (13b)

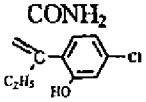
(49%) (~5%)

Pawar and Borse[9] reported 4-n-alkyl substituted pyrazoles (**15 a-f**) from phenyl hydrazones (**14 a-d**), semicarbazone (**14e**) and azine of 2-n-acyl-5-chlorophenol (**14f**) by monoformylation and cyclization by using one mole of the Vilsmeier-Haack reagent (DMF-$POCl_3$).

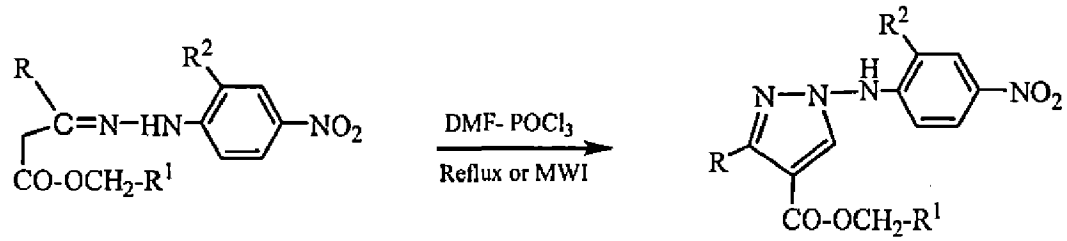


(14) (15)

	R^1	R^2
(a)	CH_3	Ph
(b)	C_2H_5	Ph
(c)	(CH_2)-CH_3	Ph
(d)	$(CH_2)_2$-CH_3	Ph
(e)	CH_3	$CONH_2$
(f)	CH_3	

	R^1	R^2
(a)	CH_3	Ph
(b)	C_2H_5	Ph
(c)	(CH_2)-CH_3	Ph
(d)	$(CH_2)_2$-CH_3	Ph
(e)	CH_3	$CONH_2$
(f)	CH_3	

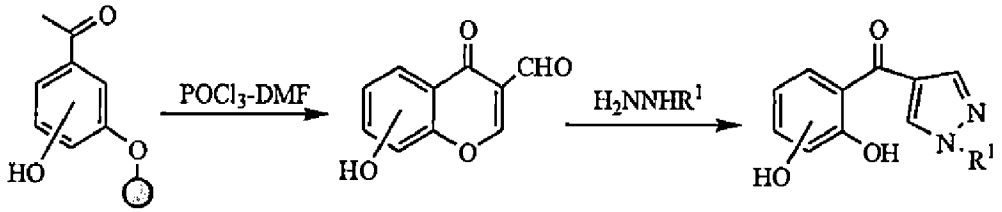
Sridhar[10] reported pyrazole-4-carboxaldehyde (17 a-h) by the reaction of hydrazones of aliphatic and aromatic methylketones (16 a-h) with Vilsmeier reagent. They also studied the reactivity of hydrazones of β-ketoesters towards Vilsmeier reagent, both by conventional and microwave methods.



(16) (17)

R	R^1	R^2	R	R^1	R^2
(a) CH_3	H	H	(a) CH_3	H	H
(b) CH_3	CH_3	H	(b) CH_3	CH_3	H
(c) CH_3	H	NO_2	(c) CH_3	H	NO_2
(d) CH_3	CH_3	NO_2	(d) CH_3	CH_3	NO_2
(e) C_6H_5	CH_3	H	(e) C_6H_5	CH_3	H
(f) C_6H_5	CH_3	NO_2	(f) C_6H_5	CH_3	NO_2
(g) p-ClC_6H_4	CH_3	H	(g) p-ClC_6H_4	CH_3	H
(h) p-ClC_6H_4	CH_3	NO_2	(h) p-ClC_6H_4	CH_3	NO_2

Borrell *et al.*[11] synthesized pyrazole library by using Merrifield resin as a solid-phase support to a hydroxyacetophenone (18), Vilsmeier-Haack formylation on methyl group and cyclization with substituted hydrazine to afford 4-hydroxybenzoyl-1-substituted pyrazoles (20 a-e).



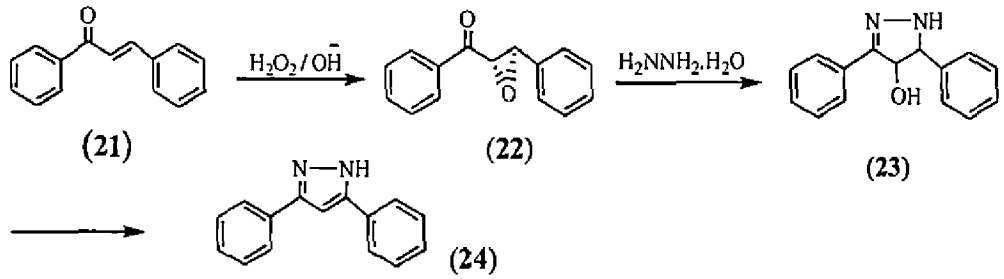
(18) (19) (20)

R^1

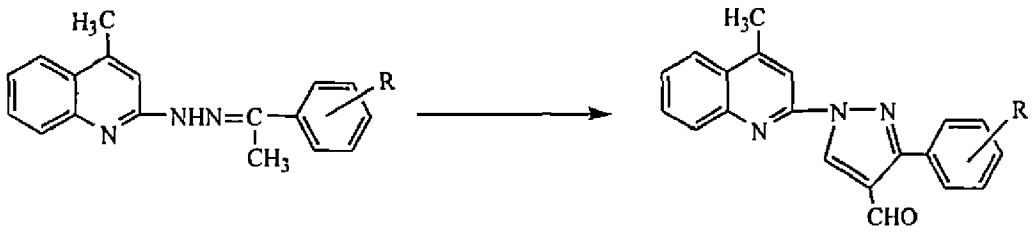
(a) H
(b) C_6H_5
(c) C_2H_5
(d) C_2H_4OH
(e) C_2H_4CN

Dhar and Bhat[12] reported the synthesis of 3, 5-diphenyl-1H-pyrazole (24) from chalcone (21) by the action of hydrazine hydrate on chalcone-epoxide (22) followed by

simultaneous dehydration in presence of catalytic amount of concentrated sulfuric acid in acetic acid.



Kidwai and coworkers[13] used microwave irradiation and synthesized 4-methyl-2-[3'-substituted-phenyl-4'-formylpyrozolyl] quinoline (**26 a-d**) by the reaction of hydrazones of methylquinoline (**25 a-d**) with DMF and $POCl_3$ under microwave irradiation of 3-4 min.

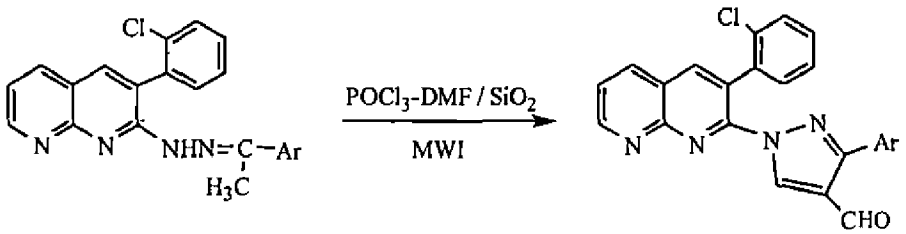


(25) (26)

R **R**

(a) H (a) H
(b) 4-Cl (b) 4-Cl
(c) 4-CH_3 (c) 4-CH_3
(d) 4-Br (d) 4-Br

3-Aryl-4-formyl-1-[3(2-chlorophenyl)-1, 8-naphthyridin-2-yl] pyrazoles (**28 a-d**) were obtained when hydrazones (**27 a-d**) were subjected to microwave irradiation[14] in the presence of Vilsmeier-Haack reagent.

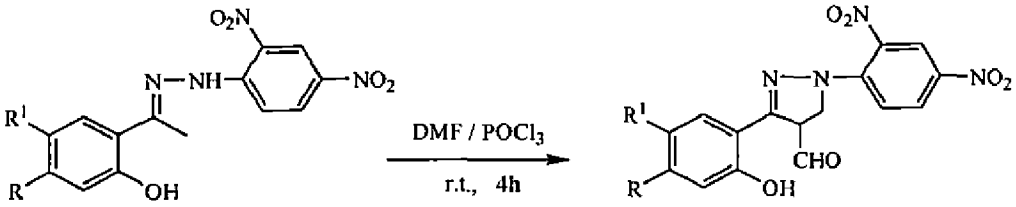


(27) (28)

Ar **Ar**

(a) C_6H_5 (a) C_6H_5
(b) 4-CH_3-C_6H_4 (b) 4-CH_3-C_6H_4
(c) 4-CH_3O-C_6H_4 (c) 4-CH_3O-C_6H_4
(d) 4-ClC_6H_4 (d) 4-ClC_6H_4

Selvi and Perumul [15] synthesized 4-ethoxy-4H-benzopyrano [4, 3 - *c*] pyrazoles (**30 a-f**) from *o*-hydroxyacetophenone-2, 4-dinitrophenylhydrazone (**29 a-f**) under Vilsmeier conditions.

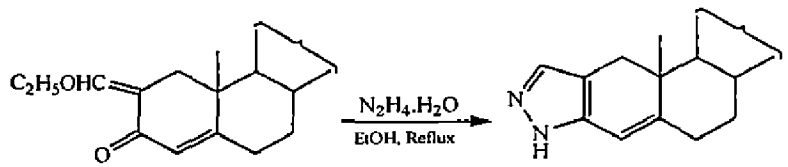


(29) (30)

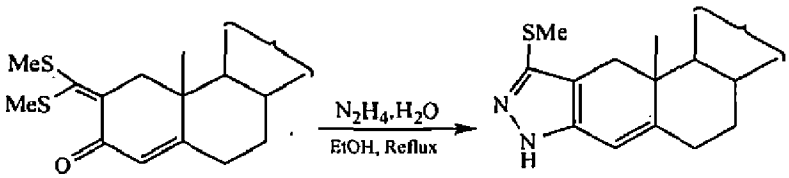
R	R^1
(a) H	H
(b) H	OCH_3
(c) H	CH_3
(d) H	Cl
(e) H	OH
(f) OCH_3	H

R	R^1
(a) H	H
(b) H	OCH_3
(c) H	CH_3
(d) H	Cl
(e) H	OH
(f) OCH_3	H

Singh *et al.*[16] reported the synthesis of pyrazolo [3, 2 - *c*] cholest-4-ene (**32**) and [1′ H]-5′-(methylthio) pyrazolo [3, 2 - *c*] cholest-4-ene (**34**) from 2-ethoxymethylene-4-cholest-4-en-3-one (**31**) and bis (methylthio) methylene cholest-4-en-3-one (**33**), respectively by the action of hydrazine hydrate in ethanol under reflux.

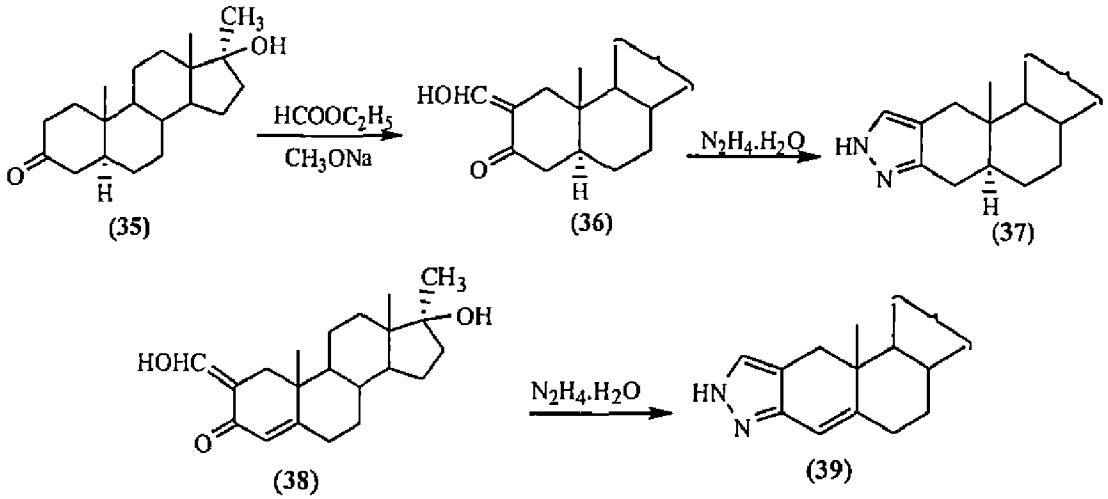


(31) (32)

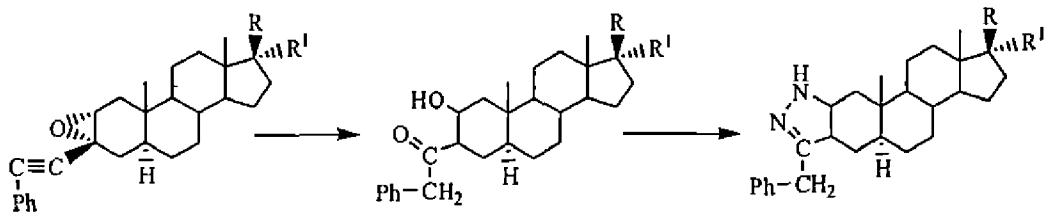


(33) (34)

Clinton *et al.*[17] reported that the treatment of 17α-methylandrostan-17β-ol-3-one (**35**) with ethylformate and sodium methoxide gave 2-hydroxymethylene derivative (**36**) which on condensation with hydrazine gave 17β-hydroxy-17α-methylandrostano [3, 2 - *c*] pyrazole (**37**). Similar treatment of 2-hydroxymethylene-17α-androst-4-en-17β-ol-3-one (**38**) furnished 17β-hydroxy-17α-methylandrost-4-eno [3, 2 - *c*] pyrazole (**39**).

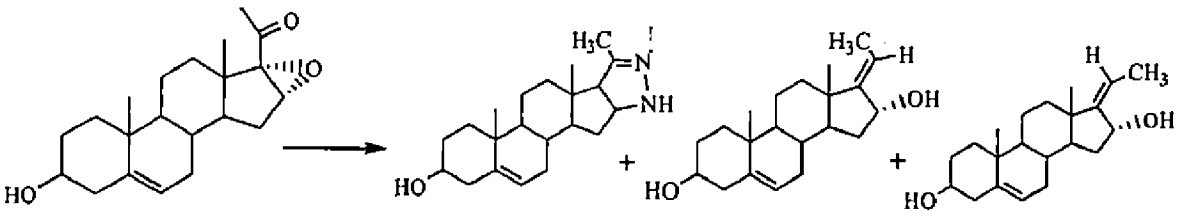


Berbalk and coworkers[18] reported that the epoxyandrostane (**40 a-c**) underwent formolysis to give phenyl acetylandrostane (**41 a-c**) which on further cyclocondensation with hydrazine afforded steroidal pyrazoles (**42 a-c**).

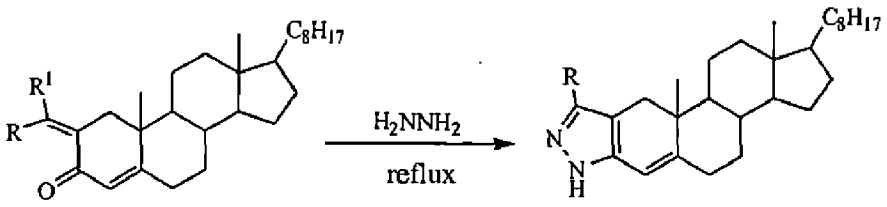


(40)		(41)		(42)	
R	**R¹**	**R**	**R¹**	**R**	**R¹**
(a) OAc	H	(a) OAc	H	(a) OAc	H
(b) OH	H	(b) OH	H	(b) OH	H
(c) R, R¹	=O	(c) R, R¹	=O	(c) R, R¹	=O

Bonn and Dodson[19] carried out the hydrazine reduction of 16α, 17-epoxy-pregnenolone (**43**) to obtain 3β-hydroxyandrost-5-eno [16, 17- *c*]-5-methylpyrazole (**44**) along with the two isomeric allylic alcohols, 5, 17 [20]-(*cis*)-pregnadiene-3β, 16α-diol (**45a**) and 5, 17 [20]-(*trans*)-pregnadiene-3β, 16α-diol (**45b**).



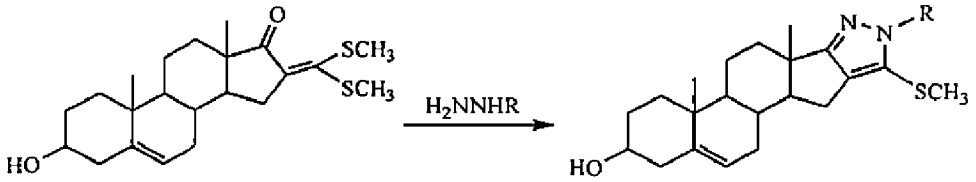
Laitonjan and co-workers[20] synthesized substituted steroidal [3, 2 - *c*] pyrazoles (**47 a, b**) by the reaction of 2-ethoxymethylene-4-cholestan-3-one (**46a**) and 2-bis (methylthio) methylene cholest-4-en-3-one (**46b**) with hydrazine hydrate and refluxed for 3 h.



(46) (47)

R	R^1	R
(a) H	OC_2H_5	(a) H
(b) SCH_3	SCH_3	(b) SCH_3

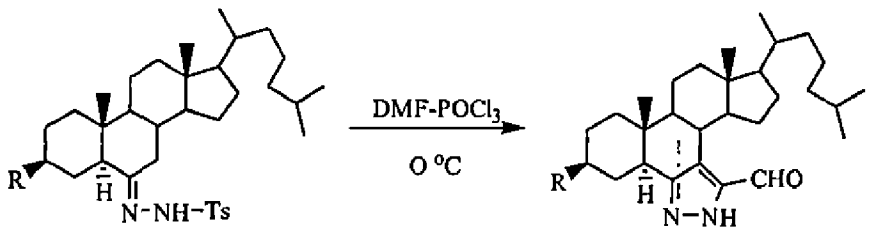
Peseke *et al.*[21] reported that the treatment of 3*β*-acetoxy-16-[bis (methylthio) methylene]-5-androst-5-en-17-one (**48**) with hydrazine hydrate and methylhydrazine afforded the 5-methylthio-pyrazolo [4′, 3′ : 16, 17] androst-5-en-3*β*-ols (**49 a, b**).



(48) (49)

R
(a) H
(b) CH_3

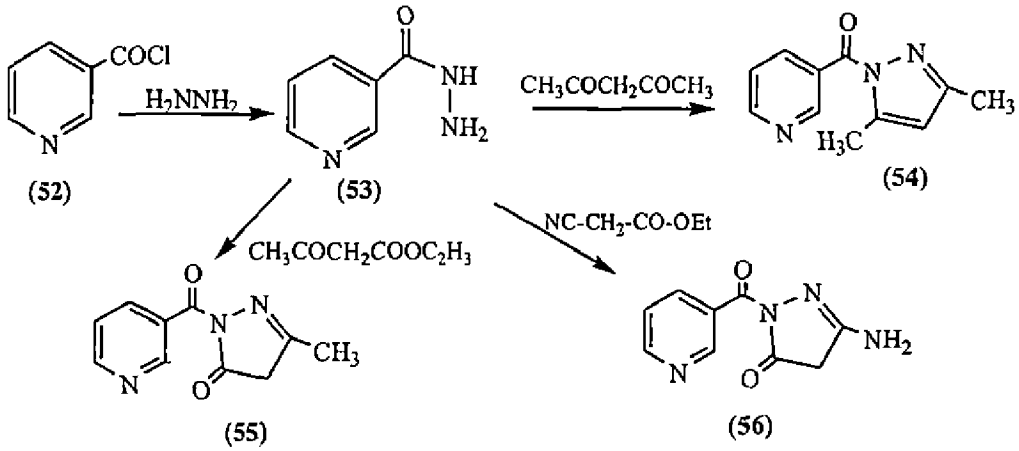
Shamsuzzaman *et al.*[22] reported that the treatment of 5*α*-cholestan-6-one tosylhydrazones (**50 a-c**) with Vilsmeier reagent gave 5′-formyl-5*α*-cholestan [6, 7 - *c*] pyrazole derivatives (**51 a-c**) in 60-65% yields.



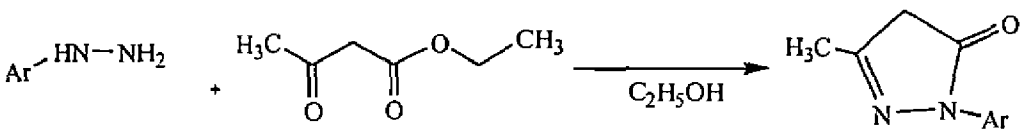
(50) (51)

R	R
(a) H	(a) H
(b) OAc	(b) OAc
(c) Cl	(c) Cl

Sidhaye *et al.*[23] reported that nicotinoyl chloride (**52**) reacted with hydrazine and gave nicotinohydrazide (**53**). The compound (**53**) on reaction with acid derivatives yielded (3, 5-dimethyl-1H-pyrazol-1-yl)(pyridine-3-yl) methanone (**54**), 3-methyl-1-nicotinoyl-1H-pyrazol-5(4H)-one (**55**), 3-amino-1-nicotinoyl-1H-pyrazol-5(4H)-one (**56**) in good amounts.

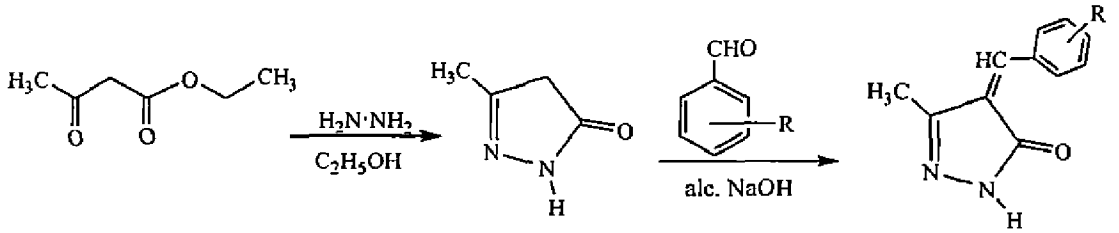


Isloor and co-workers[24] reported the reaction of substituted hydrazines (**57 a-g**) with ethyl acetoacetate in absolute alcohol which provided the corresponding substituted pyrazolones (**58 a-g**) in good yields.



Ar	**Ar**
(a) C_6H_5	(a) C_6H_5
(b) 2, 4-DNP	(b) 2, 4-DNP
(c) 4-Chlorophenyl	(c) 4-Chlorophenyl
(d) 4-OCH_3-C_6H_4	(d) 4-OCH_3-C_6H_4
(e) Biphenyl	(e) Biphenyl
(f) 2, 4-Dichlorophenyl	(f) 2, 4-Dichlorophenyl
(g) 4-SCH_3-C_6H_4	(g) 4-SCH_3-C_6H_4

Mariappan and co-workers[25] reported that the reaction of ethyl acetoacetate with hydrazine hydrate in absolute alcohol yielded pyrazolone (**59**). The pyrazolone (**59**) upon reaction with different substituted aromatic aldehydes in presence of alcoholic sodium hydroxide yielded corresponding substituted pyrazolones (**60 a-h**).

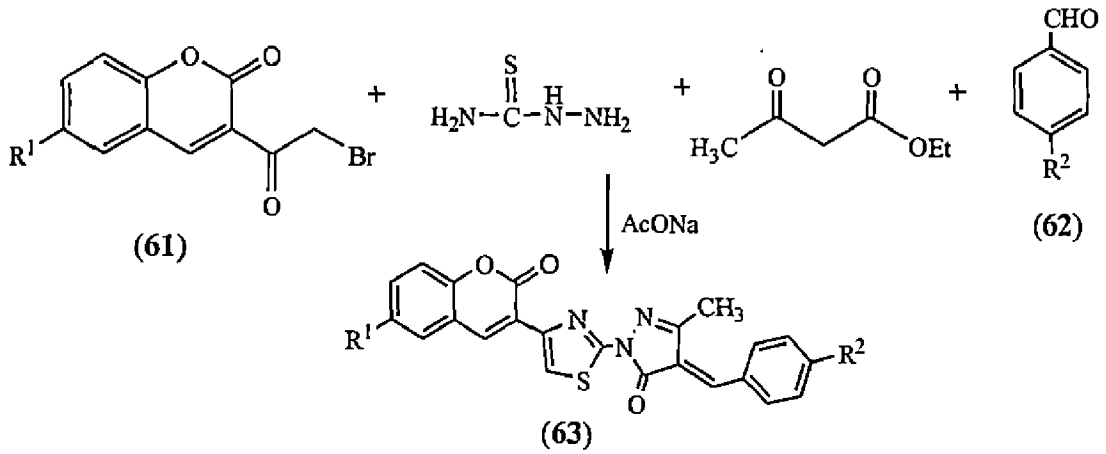


(59) (60)

R

(a) $N(CH_3)_2$ (e) 3-Cl
(b) Ph-CH=CH (f) 4-OMe
(c) $OCH_{3\,(2,3)}$ (g) 3-OMe
(d) 3-NO_2 (h) OH

Rao and Chunduru[26] reported the one pot synthesis of 4-arylidene-3-methyl-1-[4-(2-oxo-2H-chromen-3-yl) thiazol-2-yl]-1H-pyrazol-5(4H)-ones (**63 a-d**) by reacting 3-(2-bromoacetyl) coumarin derivatives (**61 a-d**), thiosemicarbazide, ethyl acetoacetate and substituted aryl aldehydes (**62 a-d**) in presence of sodium acetate in acetic acid.



(61) (62) (63)

(61)

R^1

(a) H
(b) Br
(c) NO_2
(d) OCH_3

(62)

R^2

(a) NH_2
(b) NMe_2
(c) NO_2
(d) NHMe

(63)

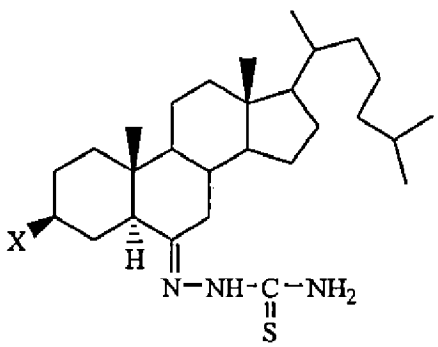
R^1	**R^2**
(a) H	NH_2
(b) Br	NMe_2
(c) NO_2	NO_2
(d) OCH_3	NHMe

Discussion

As a part of our continuing effort towards the synthesis of heterosteroids, which are expected to be biologically active, the fusion of heterocycles to steroids often lead to a change in physiological activities or appearance of new interesting biological behavior.[27] Thus, several steroid appended heterocycles have been synthesized which act as potential inhibitors of cytochrome P450 aromatage[28, 29] with their subsequent clinical applications in the treatment of estrogen dependent breast cancer.

Pyrazole moiety being called as pharmacophore plays an essential role in biologically active compounds and therefore represents an interesting template for combinatorial as well as medicinal chemistry.[30, 31] In addition pyrazoles have played a vital role in the development of theory in heterocyclic chemistry and are also used extensively as useful synthons in organic synthesis.[32] These derivatives have wide spread potential biological activities such as antimicrobial, anticancer, antioxidant, analgesic, anti-inflammatory, antipyretic, antiviral, anticonvulsant, antihistaminic, antidepressant, insecticidal and anti-HIV.[33-38] The recent success of pyrazole COX-2 inhibitor has further highlighted the importance of these heterocycles in medicinal chemistry.

The therapeutic importance of these steroidal pyrazoles[16] and study of interesting behavior of Vilsmeier reagent with simple hydrazones giving pyrazoles encouraged us to make similar studies with steroidal thiosemicarbazones. The substrates selected for synthesizing the new steroidal pyrazoles include 5α-cholestan-6-one thiosemicarbazone[39] (**64**), 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone[39] (**65**) and 3β-chloro-5α-cholestan-6-one thiosemicarbazone[39] (**66**). The products obtained have been characterized on the basis of spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and elemental analyses.

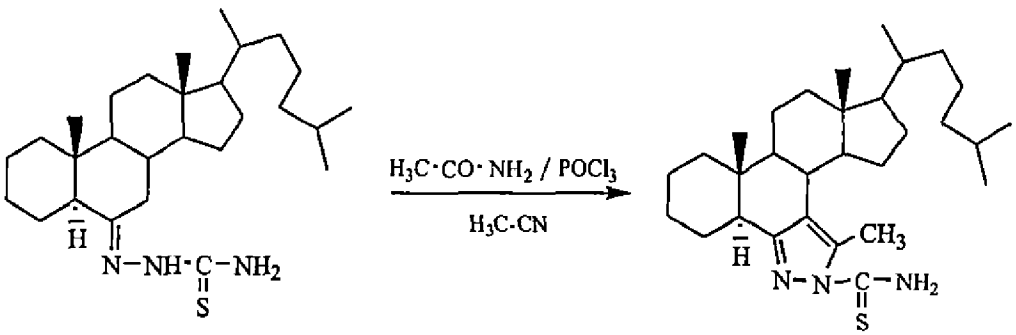


X

(**64**) H
(**65**) OAc
(**66**) Cl

Reaction of 5α-cholestan-6-one thiosemicarbazone (64) with $POCl_3$ and acetamide.

The 5α-cholestan-6-one thiosemicarbazone (64) in CH_3CN was allowed to react with $POCl_3$ and acetamide. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **67**, m.p 121 °C.



(64) **(67)**

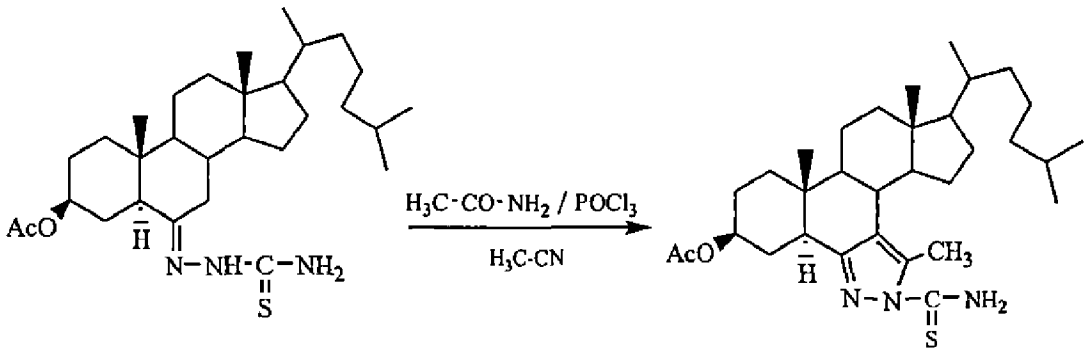
Characterization of the compound, m.p. 121 °C as 5α-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole (67):

The elemental analysis of compound **67** corresponded to the molecular formula $C_{30}H_{49}N_3S$. Its IR spectrum showed a band at 3393 cm^{-1} which could be assigned to NH group while as the bands at 1652, 1620, 1375 and 1275 cm^{-1} were attributed to C=N, C=C, C-N and C=S group, respectively. These values supported the presence of pyrazole moiety[40] in the product molecule. The structure **67** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 7.6 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for three protons at δ 2.4 showed the presence of C_5'-CH_3. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.19, 0.96, 0.83 and 0.75. The ^{13}C NMR spectrum of compound **67** displayed characteristic signals at δ 179.2 (*C*=S), 150.1 (C_6), 118 (C_7), 137 (C_5') and 25.2 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **67** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 483) was observed.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 3.1**), this compound can be best characterized as 5α-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole (**67**).

Reaction of 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (65) with $POCl_3$ and acetamide.

The 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone (**65**) in CH_3CN was allowed to react with $POCl_3$ and acetamide. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **68**, m.p 147 °C.



(65) (68)

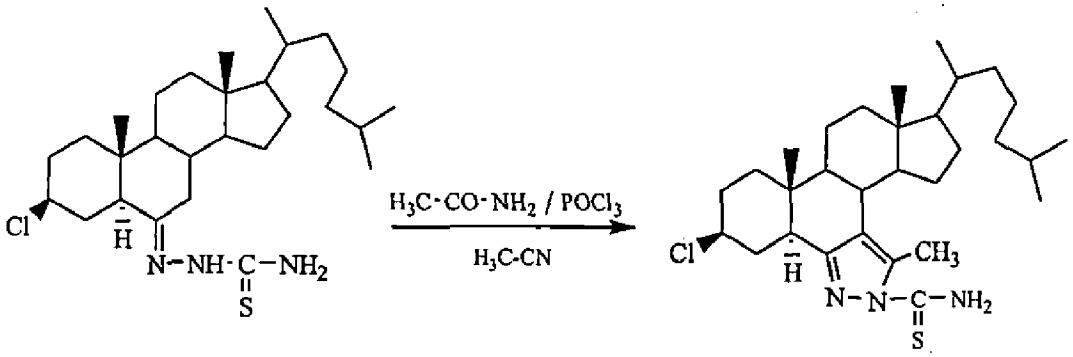
Characterization of the compound, m.p. 147 °C as 3β-acetoxy-5α-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole (68):

The compound **68** was correctly analyzed for the molecular formula $C_{32}H_{51}N_3O_2S$. Its IR spectrum showed a band at 3390 cm^{-1} which could be assigned to NH group. The strong IR absorption bands at 1714 and 1210 cm^{-1} indicated the presence of acetate group, while as the bands at 1655, 1632, 1376 and 1269 cm^{-1} were attributed to C=N, C=C, C-N and C=S group, respectively. These values confirmed the presence of pyrazole moiety[40] in the product molecule. The structure **68** was well supported by its ^1H NMR spectrum which displayed a broad singlet integrating for two protons at δ 8.2 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for three protons at δ 2.3 showed the presence of C_5'-CH_3. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) was observed at δ 4.7 for one proton which could be assigned to $C_3\alpha$-*H*. The three acetate group protons appeared at δ 2.03 as a sharp singlet. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound **68** displayed characteristic signals at δ 181.2 (*C*=S), 148.3 (C_6), 119 (C_7), 134 (C_5') and 75.1 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **68** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 541) was found.

On the basis of above studies and its analogy with earlier compound **67**, this compound can be best characterized as 3*β*-acetoxy-5*α*-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole (**68**).

Reaction of 3*β*-chloro-5*α*-cholestan-6-one thiosemicarbazone (66) with $POCl_3$ and acetamide.

The 3*β*-chloro-5*α*-cholestan-6-one thiosemicarbazone (**66**) in CH_3CN was allowed to react with $POCl_3$ and acetamide. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to afford compound **69**, m.p 156 °C.



(66) (69)

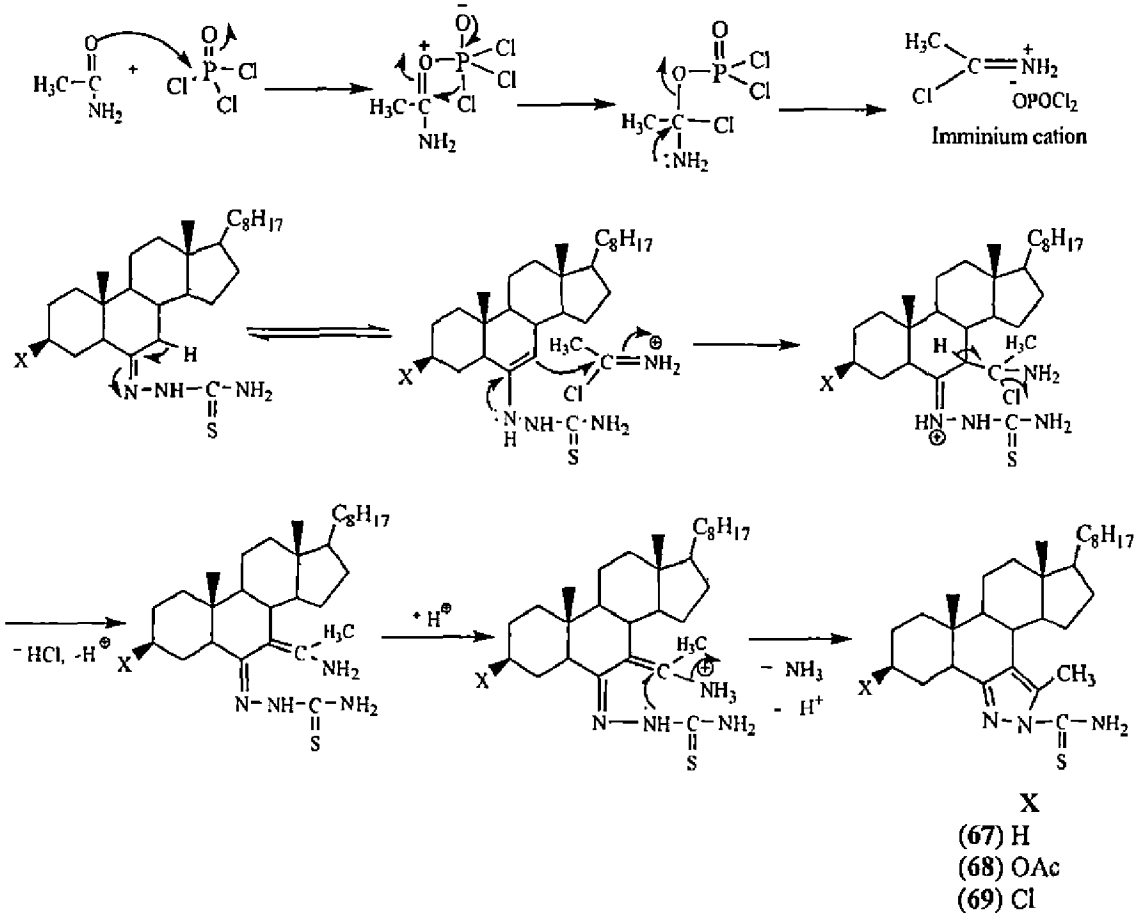
Characterization of the compound, m.p. 156 °C as 3*β*-chloro-5*α*-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole (69):

The elemental analysis of compound **69** corresponded to the molecular formula $C_{30}H_{48}N_3ClS$ (Beilstein positive). Its IR spectrum showed a band at 3410 cm^{-1} which could be assigned to NH group while as the bands at 1650, 1625, 1378, 1275 and 756 cm^{-1} were attributed to C=N, C=C, C-N, C=S and C-Cl group, respectively. These values supported the presence of pyrazole moiety[40] in the product molecule. The structure **69** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 7.8 (exchangeable with D_2O) indicating the presence of NH_2 while as the singlet integrating for three protons at δ 2.4 showed the presence of C_5'-CH_3. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. Angular and side-chain methyl protons were observed at δ 1.19, 0.97, 0.75 and 0.80. The ^{13}C NMR spectrum of compound **69** displayed characteristic signals at δ 184.2 (*C*=S), 144.5 (C_6), 119.2 (C_7), 132

(C_5') and 52.6 (C_3). Remaining carbon atoms were seen as per the cholestane series. The structure of compound **69** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 515/517) was observed.

The above data led to the structure of compound **69** as, 3*β*-chloro-5*α*-cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole.

Formation of steroidal pyrazoles (**67-69**) under the condition case and in the light of available literature [15, 22] may be shown according to the proposed mechanism (**Scheme 3.1**).



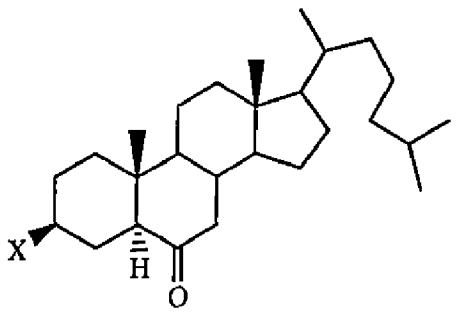
Scheme 3.1 Mechanism for the formation of cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole derivatives (**67-69**)

Work communicated;

DNA binding studies and *in vitro* cytotoxicity of newly synthesized steroidal pyrazoles, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., (communicated)

Pyrazolones have gained importance as drug substances in pharmaceutical industry in view of their biological importance. For instance, the pyrazolones, *viz.* phenazone, propyphenazone, ampyrone and metamizole are useful antipyretic and analgesic drugs,[41] whilst edaravone (MCI-186) has been used for treating brain[42] and myocardial ischemia.[43] In addition, pyrazolones possess antimicrobial, antimycobacterial, anti-inflammatory, antitumor, gastric secretion stimulatory, antidepressant and antifilarial activities.[44-50] They also serve as precursors for the synthesis of dyes, pigments, pesticides and chelating agents.[51] Besides finding applications in the extraction and separation of various metal ions,[52] they are also employed in chromatography, petrochemical industry, as laser materials and ^{1}H NMR shift reagents.[53, 54]

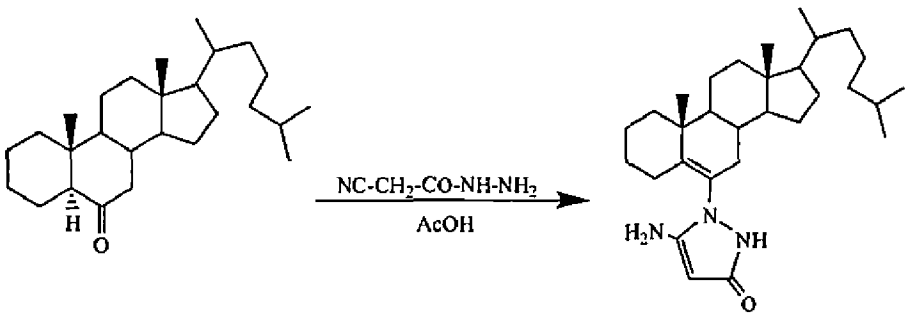
The substrates selected for synthesizing the new steroidal pyrazolones (**73-75**) include 5α-cholestan-6-one[55] (**70**), 3β-acetoxy-5α-cholestan-6-one[56] (**71**) and 3β-chloro-5α-cholestan-6-one[57] (**72**). The products obtained have been characterized on the basis of spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and elemental analyses.



X
(**70**) H
(**71**) OAc
(**72**) Cl

Reaction of 5α-cholestan-6-one (70) with cyanoacetohydrazide.

The 5α-cholestan-6-one (**70**) in acetic acid (15 mL) was allowed to react with cyanoacetohydrazide in equimolar ratio. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water, 5% $NaHCO_3$, again with water and dried over anhydrous sodium sulfate. Removal of the solvents provided crude product which was recrystallized from methanol to afford compound **73**, m.p 119 °C.



(70) (73)

Characterization of the compound, m.p. 119 °C as cholest-6[5′-amino-1′, 2′-dihydro pyrazol-3-one-1′-yl] 5-ene (73):

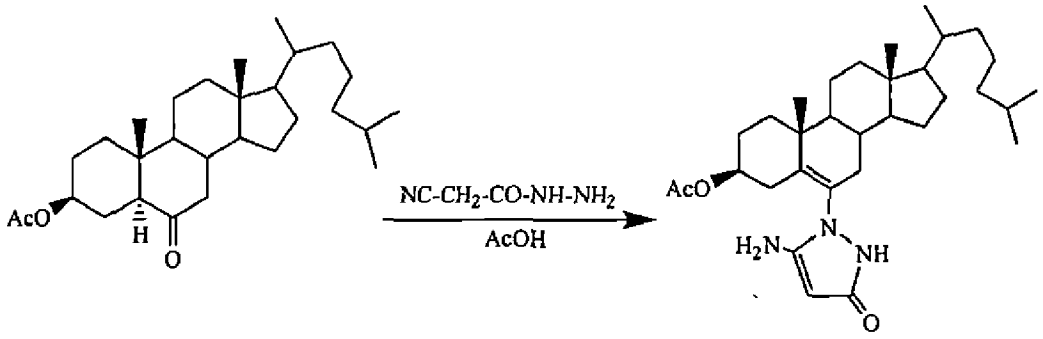
The elemental analysis of compound **73** corresponded to the molecular formula $C_{30}H_{49}N_3O$. Its IR spectrum showed bands at 3470 and 3296 cm^{-1} which could be assigned to NH and NH_2 group while as the bands at 1680, 1620/1624, 1441 and 1461 cm^{-1} were attributed to *CO*NH, C=C, C-N and N-N group, respectively. These values supported the presence of pyrazolone moiety[40] in the product molecule. The structure **73** was well supported by its ^1H NMR spectrum in which a singlet at δ 8.8 (exchangeable with D_2O) was assigned for one proton (N*H*) while as the broad singlet at δ 2.1 (exchangeable with D_2O) depicted the presence of two protons (NH_2). Another singlet appeared at δ 5.6 showed the presence of an olefinic proton (C_4'-*H*) in the compound. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.12, 0.92, 0.85 and 0.71. The ^{13}C NMR spectrum of the compound **73** displayed characteristic signals at δ 171 (C_3'), 151 (C_5'), 132 (C_6), 117 (C_5), 127 (C_4') and 22.3 (C_3). Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **73** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 467) was found.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 3.2**), this compound can be best characterized as cholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene (73).

Reaction of 3β-acetoxy-5α-cholestan-6-one (71) with cyanoacetohydrazide.

The 3β-acetoxy-5α-cholestan-6-one (71) in acetic acid (15 mL) was allowed to react with cyanoacetohydrazide in equimolar ratio. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water, 5% $NaHCO_3$, again with water and

dried over anhydrous sodium sulfate. Removal of the solvents gave crude product which was recrystallized from methanol to afford compound **74**, m.p 145 °C.



(71) **(74)**

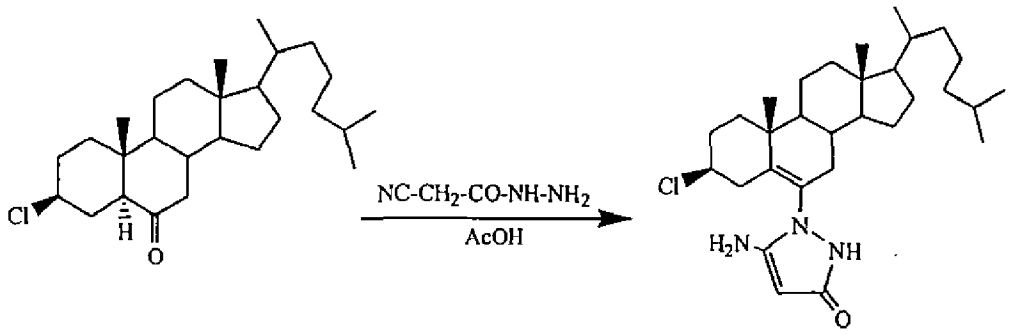
Characterization of the compound, m.p. 145 °C as 3β-acetoxycholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene (74):

The compound **74** was correctly analyzed for the molecular formula $C_{32}H_{51}N_3O_3$. Its IR spectrum showed bands at 3345 and 3280 cm^{-1} which could be assigned to NH and NH_2 group. The strong absorption bands at 1735 and 1210 cm^{-1} indicated the presence of acetate group, while as the bands at 1680, 1615/1625, 1455 and 1480 cm^{-1} were attributed to *C*ONH, C=C, C-N and N-N group, respectively. These values suggested the presence of pyrazolone moiety[40] in the product molecule. The structure **74** was well supported by its ^1H NMR spectrum which showed a singlet at δ 9.0 (exchangeable with D_2O) depicted the presence of N*H* while as the broad singlet at δ 2.2 (exchangeable with D_2O) showed the presence of two protons (NH_2). A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. The three acetoxy group protons appeared at δ 2.01 as a sharp singlet. Another singlet appeared at δ 5.2 revealing the presence of an olefinic proton (C_4'-*H*) in the compound. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.12, 0.92, 0.85 and 0.71. The ^{13}C NMR spectrum of compound **74** displayed characteristic signals at δ 171 (*C*O), 176 (C_3'), 159 (C_5'), 135 (C_6), 119 (C_5), 123 (C_4') and 70.2 (C_3). Remaining carbon atoms were seen as per the cholestane series. The structure of compound **74** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 525) was observed.

On the basis of above studies and its analogy with earlier compound **73**, this compound can be best characterized as 3β-acetoxycholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene (**74**).

Reaction of 3β-chloro-5α-cholestan-6-one (72) with cyanoacetohydrazide.

The 3β-chloro-5α-cholestan-6-one (72) in acetic acid (15 mL) was allowed to react with cyanoacetohydrazide in equimolar ratio. After completion of the reaction, the reaction mixture was taken in diethyl ether, washed with water, 5% $NaHCO_3$, again with water and dried over anhydrous sodium sulfate. Removal of the solvents provided crude product which was recrystallized from methanol to afford compound **75**, m.p 131 °C.



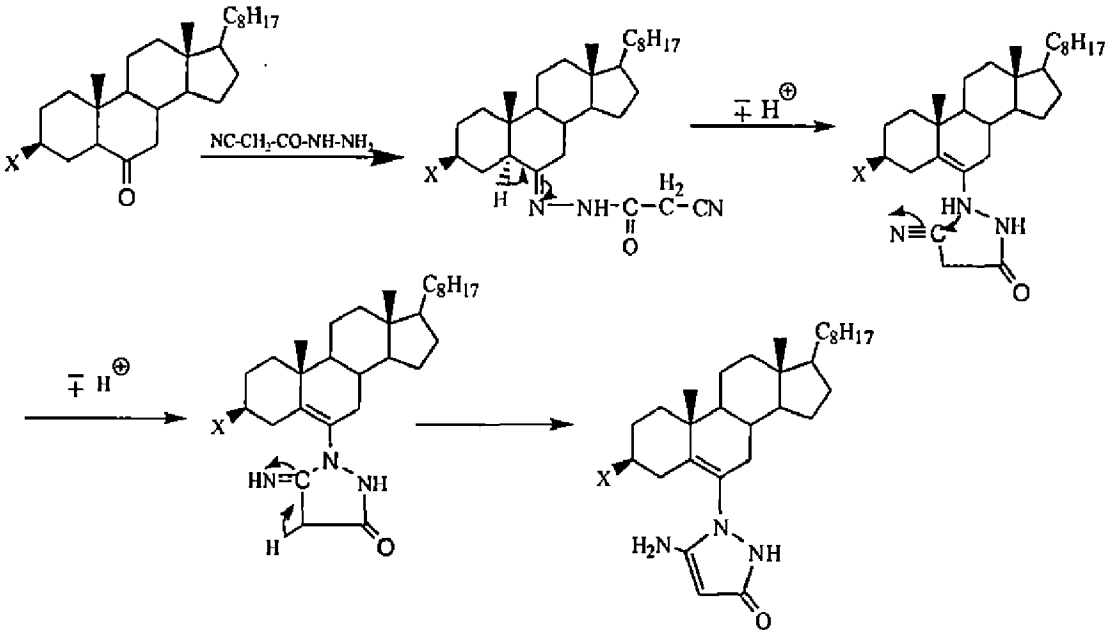
(72) (75)

Characterization of the compound, m.p. 131 °C as 3β-chlorocholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene (75):

The elemental analysis of compound **75** corresponded to the molecular formula $C_{30}H_{48}ClN_3O$ (Beilstein positive). Its IR spectrum showed bands at 3375 and 3291 cm^{-1} which ɔuld be assigned to NH and NH_2 group while as the bands at 1685, 1618/1622, 1448, 1467 741 cm^{-1} were attributed to *CO*NH, C=C, C-N, N-N and C-Cl group, respectively. These ; supported the presence of pyrazolone moiety[40] in the product molecule. The structure ⁻ well supported by its ^1H NMR spectrum which showed one proton singlet at δ 8.5 geable with D_2O) suggesting the presence of N*H* while a two-proton broad singlet at δ hangeable with D_2O) depicted the presence of NH_2. A broad multiplet ($W_{1/2}$ = 17 Hz, ɔr one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. Another singlet d at δ 5.4 indicating the presence of an olefinic proton (C_4'-*H*) in the compound. The ɔnt peaks for angular and side-chain methyl protons were observed at δ 1.12, 0.92, ł 0.71. The ^{13}C NMR spectrum of compound **75** displayed characteristic signals at δ '), 154.7 (C_5'), 136 (C_6), 117 (C_5), 120 (C_4') and 52.6 (C_3). Remaining carbon atoms en in accordance to the cholestane series. The structure of compound **75** was further ɛd by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 499/501) was

The above data led to the structure of compound **75** as, 3β-chlorocholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene.

Formation of steroidal pyrazolones (**73-75**) under the condition case and in the light of available literature[58] may be shown according to the proposed mechanism (**Scheme 3.2**). The mechanism for the formation of these pyrazolones involve the simple condensation thus formation of 5α-cholestan-6-one cyanoacetohydrazone takes place first which then undergoes the nucleophilic attack of nitrogen on the terminal carbon (C≡N) of cyanoacetohydrazone to get converted to C=NH which later changes into C-NH_2 leads to the cyclization of heterocycle.



X

(**73**) H
(**74**) OAc
(**75**) Cl

Scheme 3.2 Mechanism for the formation of cholest-6[5′-amino-1′, 2′-dihydropyrazol-3-one-1′-yl] 5-ene derivatives (**73-75**)

Work published;

Structural, optical and antimicrobial studies of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Taibah University for Science*, http://dx.doi.org/10.1016/j.jtusci.2013.08.003 (in press)

Experimental

All the melting points were determined in degrees Celsius on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Perkin Elmer RXI Spectrophotometer and values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of boiling point 60-80 °C. Sodium sulfate (anhydrous) was used as a drying agent.

The synthesis of 3β-chlorocholest-5-ene, cholest-5-ene, 6-nitrocholest-5-ene, 5α-cholestan-6-one and 5α-cholestan-6-one thiosemicarbazone is shown in **chapter 1**, page 23 and 24. The synthesis of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene, 3β-acetoxy-5α-cholestan-6-one and 3β-acetoxy-5α-cholestan-6-one thiosemicarbazone is shown in **chapter 1**, page 24, 25 while as the synthesis of 3β-chloro-6-nitrocholest-5-ene, 3β-chloro-5α-cholestan-6-one and 3β-chloro-5α-cholestan-6-one thiosemicarbazone is shown in **chapter 1**, page 25 and 26.

Reaction of 5α-cholestan-6-one thiosemicarbazone derivatives (64-66) with $POCl_3$ and acetamide:

To a solution of 5α-cholestan-6-one thiosemicarbazone derivatives (**64-66**) (1 mmol) in CH_3CN (10 mL), was added acetamide (1 mmol) under ice-cold condition. $POCl_3$ (1 mmol) was then added at such a rate that the temperature of the reaction mixture did not exceed 10 ^{o}C with constant stirring. After the complete addition, the reaction mixture was allowed to attain room temperature; however, the stirring was continued for an additional period of 3 h. The progress of reaction was followed by TLC. After completion of the reaction, the reaction mixture was poured onto crushed ice, left overnight in a refrigerator and was extracted with diethyl ether. The ethereal layer was further washed with water and dried over anhydrous sodium sulfate. Removal of solvents gave an oil which was crystallized from methanol to give corresponding steroidal pyrazoles (**67-69**).

5α-Cholestano [6, 7 - c] 5'-methyl-1'-carbothioic acid amido pyrazole (67):

Yield 70%; m.p. 121 °C; Analysis found: C 74.53, H 10.14, N, 8.69%. $C_{30}H_{49}N_3S$ requires: C 74.40, H 10.07, N 8.60%; IR (KBr): ν_{max} 3393 (NH), 1652 (C=N) 1620 (C=C), 1375 (C-N),

T-4041

1275 (C=S); ^{1}H NMR ($CDCl_3$): δ 7.6 (s, 2H, NH_2, exchangeable with D_2O), 2.4 (s, 3H, C_5'-CH_3), 1.19 (s, 3H, C_{10}-CH_3), 0.75 (s, 3H, C_{13}-CH_3), 0.96 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 179.2 (*C*=S), 150.1 (C_6), 137 (C_5'), 118 (C_7), 41.8 (C_5), 42.76 (C_9), 39.45 (C_{10}), 36.53 (C_{13}), 25.2 (C_3); MS: *m/z* 483 [$M^{+\cdot}$].

3β-Acetoxy-5α-cholestano [6, 7 - c] 5'-methyl-1'-carbothioic acid amido pyrazole (68):

Yield 70%; m.p. 147 °C; Analysis found: C 70.97, H 9.42, N 7.76%. $C_{32}H_{51}N_3O_2S$ requires: C 70.89, H 9.19, N 7.55%; IR (KBr): ν_{max} 3390 (NH), 1714 (CH_3COO), 1655 (C=N), 1632 (C=C), 1376 (C-N), 1269 (C=S), 1210 (C-O); ^{1}H NMR ($CDCl_3$): δ 8.2 (s, 2H, NH_2, exchangeable with D_2O), 4.7 (m, 1H, $C_3\alpha$-*H*, *W* ½ = 15 Hz), 2.3 (s, 3H, C_5'-CH_3), 2.03 (s, 3H, $OCOCH_3$), 1.18 (s, 3H, C_{10}-CH_3), 0.70 (s, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 181.2 (*C*=S), 171.0 (CH_3COO), 148.3 (C_6), 134 (C_5'), 119.3 (C_7), 75.1 (C_3), 40.5 (C_5), 39.52 (C_{10}), 36.70 (C_{13}); MS: *m/z* 541 [$M^{+\cdot}$].

3β-Chloro-5α-cholestano [6, 7 - c] 5'-methyl-1'-carbothioic acid amido pyrazole (69):

Yield 65%; m.p. 156 °C; Analysis found: C 69.63, H 9.28, N 8.12%. $C_{30}H_{48}N_3ClS$ requires: C 69.49, H 9.11, N 8.02%; IR (KBr): ν_{max} 3410 (NH), 1650 (C=N), 1625 (C=C), 1378 (C-N), 1275 (C=S), 756 (C-Cl); ^{1}H NMR ($CDCl_3$): δ 7.8 (s, 2H, NH_2, exchangeable with D_2O), 3.9 (m, 1H, $C_3\alpha$-*H*, *W*½ = 17 Hz), 2.4 (s, 3H, C_5'-CH_3), 1.19 (s, 3H, C_{10}-CH_3), 0.75 (s, 3H, C_{13}-CH_3), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 184.2 (*C*=S), 144.5 (C_6), 132 (C_5'), 119.2 (C_7), 52.6 (C_3), 42.2 (C_5), 42.92 (C_5), 39.32 (C_{10}), 36.31 (C_{13}); MS: *m/z* 515/517 [$M^{+\cdot}$].

Reaction of 5α-cholestan-6-one derivatives (70-72) with cyanoacetohydrazide:

To a solution of 5α-cholestan-6-one derivatives (**70-72**) (1 mmol) in acetic acid (15 mL) was added cyanoacetohydrazide in equimolar ratio in same solvent. The reaction mixture was refluxed for 5 h. The progress of reaction was monitored by TLC. After completion of the reaction, the excess solvent was reduced to three fourths of the original volume under reduced pressure. The reaction mixture was washed with water (30 mL), neutralized with saturated aqueous $NaHCO_3$ and then taken in diethyl ether. The ethereal layer was further washed with water and dried over anhydrous sodium sulfate. Removal of solvent gave the crude product which was recrystallized from methanol to furnish corresponding steroidal pyrazolones (**73-75**).

Cholest-6 [5'-amino-1', 2'-dihydropyrazol-3-one-1'-yl]5-ene (73):

Yield 70%; m.p. 119 °C; Analysis found: C 77.08, H 10.49, N 8.99. $C_{30}H_{49}N_3O$ requires: C 77.01, H 10.41, N 8.63; IR (KBr): ν_{max} 3470, 3296 (NH, NH_2), 1680 (*CO*NH), 1624, 1620 (C=C), 1461 (N-N), 1441 (C-N); ^{1}H NMR ($CDCl_3$): δ 8.8 (s, 1H, N*H*, exchangeable with D_2O), 5.6 (s, 1H, C_4'-*H*), 2.1 (s, 2H, NH_2, exchangeable with D_2O), 1.12 (s, 3H, C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.92 and 0.85 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 171 (C_3'), 151 (C_5'), 132 (C_6), 117 (C_5), 127 (C_4'), 22.3 (C_3), 118 (C_7), 42.76 (C_9), 39.45 (C_{10}), 36.53 (C_{13}); MS: *m/z* 467 [M^+].

3β-Acetoxycholest-6[5'-amino-1', 2'-dihydropyrazol-3-one-1'-yl]5-ene (74):

Yield 80%; m.p. 145 °C; Analysis found: C 73.14, H 9.71, N 8.0%. $C_{32}H_{51}N_3O_3$ requires: C 73.04, H 9.65, N 7.95%; IR (KBr): ν_{max} 3345, 3280 (NH, NH_2), 1735 (O*CO*CH_3), 1680 (*CO*NH), 1625, 1615 (C=C), 1210 (C-O), 1455 (C-N), 1480 (N-N); ^{1}H NMR ($CDCl_3$): δ 9.0 (s, 1H, N*H*, exchangeable with D_2O), 5.2 (s, 1H, C_4'-*H*), 4.7 (m, 1H, $C_3\alpha$-*H*, *W½* = 15 Hz), 2.2 (s, 2H, NH_2, exchangeable with D_2O), 2.01 (s, 3H,OCOCH_3), 1.12 (s, 3H,C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.92 and 0.85 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 171.1 (*CO*), 176 (C_3'), 159 (C_5'), 135 (C_6), 119 (C_5), 123 (C_4'), 70.2 (C_3), 118 (C_7), 42.76 (C_9), 39.45 (C_{10}), 36.53 (C_{13}); MS: *m/z* 525 [M^+].

3β-Chlorocholest-6[5'-amino-1', 2'-dihydropyrazol-3-one-1'-yl]5-ene (75):

Analysis found: C 72.14, H 9.61, N 8.41%. $C_{30}H_{48}ClN_3O$ requires: C 72.32, H 9.58, N 8.32%; IR (KBr): ν_{max} 3375, 3291 (NH, NH_2), 1685 (*CO*NH), 1622, 1618 (C=C), 1448 (C-N), 1467 (N-N), 741 (C-Cl); ^{1}H NMR ($CDCl_3$): δ 8.5 (s, 1H, N*H*, exchangeable with D_2O), 5.4 (s, 1H, C_4'-*H*), 3.9 (1H, m, $C_3\alpha$-*H*, *W½* = 17 Hz), 2.3 (s, 2H, NH_2, exchangeable with D_2O), 1.12 (s, 3H, C_{10}-CH_3), 0.71 (s, 3H, C_{13}-CH_3), 0.92 and 0.85 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 177 (C_3'), 154.7 (C_5'), 136 (C_6), 117 (C_5), 120 (C_4'), 52.6 (C_3), 118 (C_7), 42.76 (C_9), 39.45 (C_{10}), 36.53 (C_{13}); MS: *m/z* 499/501 [M^+].

References

References

1. L. Knorr, *Ger. Pat.*, **26**, 429 (1883)
2. L. Knorr, *Ber.*, **16**, 2597 (1883)
3. L. Knorr, A. Blank, *Ber.*, **18**, 311 (1885)
4. L. Ruzicka, P. A. Platter, *Helv. Chim. Acta*, **21**, 1717 (1938)
5. M. A. Kira, M. O. A-Raeman, K. Z. Gadalla, *Tetrahedron Lett.*, **2**, 109 (1969)
6. M. A. Kira, M. N. A-Gnein, M. I. Korkor, *J. Heterocycl. Chem.*, **7**, 25 (1970)
7. M. K. Brantenko, V. A. Chornous, M. V. Vovk, *Chem. Heterocycl. Comp.*, **37**, 467 (2001)
8. W. E. Parham, J. E. Dooley, *J. Am. Chem. Soc.*, **15**, 985 (1967)
9. R. A. Pawar, A. P. Borse, *J. Indian. Chem. Soc.*, **66**, 203 (1989)
10. R. Sridhar, P. T. Perumal, *Synth. Commun.*, **33**, 1483 (2003)
11. J. I. Borrell, E. Schuler, J. Teixido, E. L. Michelotti, *Mol. Div.*, 1 (2004)
12. B. A. Bhat, S. C. Puri, M. A. Qurishi, K. L. Dhar, G. N. Qazi, *Synth. Commun.*, **35**, 1135 (2005)
13. M. Kidwai, Y. Goel, R. Kumar, *Indian J. Chem.*, **37B**, 174 (1998)
14. K. Mogialih, S. C. Reddy, *Indian J. Chem.*, **43B**, 2010 (1998)
15. S. Selvi, P. T. Perumal, *Indian J. Chem.*, **39B**, 163 (2000)
16. R. K. T. Singh, L. W. Singh, *Indian J. Chem.*, **38B**, 847 (1999)
17. R. O. Clinton, A. J. Manson, F. W. Stonner, A. L. Beyler, G. O. Potts, A. Arnold, *J. Am. Chem. Soc.*, **81**, 1513 (1959)
18. H. Berbalk, K. Eichinger, R. Schuster, *Arch. Pharm.*, **315**, 79 (1982)
19. W. R. Benn, R. M. Dodson, *J. Org. Chem.*, **29**, 1142 (1964)
20. W. S. Laitonjam, T. S. Rajkumar, B. S. Chingakham, *Steroids*, **67**, 203 (2002)
21. D. G. Rivera, K. Pesek, T. Jommaran, A. Montero, R. Molina, F. Coll, *Molecules*, **8**, 444 (2003)
22. Shamsuzzaman, M. S. Khan, Z. Tabassum, M. Alam, *Indian J. Chem.*, **48B**, 1183 (2009)
23. R. V. Sidhaye, A. E. Dhanawade, K. Manasa, G. Aishwarya, *Curr. Pharm. Res.*, **1**, 135 (2011)
24. A. M. Vijesh, A. M. Isloor, S. Isloor, K. N. Shivananda, P. C. Shyma, T. Arulmoli, *Der Pharma Chemica*, **3**, 454 (2011)
25. G. Marriapan, B. P. Saha, L. Sutharson, A. Haider, *Indian J. Chem.*, **49B**, 1671 (2010)
26. V. S. R. Chunduru, V. R. Rao, *J. Sulfur Chem.*, **31**, 545 (2010)
27. A. Kumar, O. Prakash, N. Kinger, S. P. Singh, *Can. J. Chem.*, **84**, 438 (2006)

28. S. Li, E. J. Parish, C. Rodriguez-Valenzuela, A. H. M. Brodie, *Bioorg. Med. Chem.*, **6**, 525 (1998)
29. B. E. Fink, D. S. Mortensen, S. R. Stauffer, Z. D. Aron, J. A. Katzenellenbogen, *Chem. Biol.*, **6**, 205 (1999)
30. C. Temperini, A. Scozzafava, C. T. Supuran, *Bioorg. Med. Chem. Lett.*, **16**, 5152 (2006)
31. J. M. Fevig, J. Cacciola, J. J. Buriak, K. A. Rossi, R. M. Knabb, J. M. Luettgen, P. C. Wong, S. A. Bia, R. R. Wexler, P. Y. Lam, *Bioorg. Med. Chem. Lett.*, **16**, 3755 (2006)
32. K. P. Chirag, C. S. Rami, B. Panigrahi, C. N. Patel, *J. Chem. Pharm. Res.*, **2**, 73 (2010)
33. A. K. Tewari, A. Mishra, *Bioorg. Med. Chem.*, **9**, 715 (2001)
34. E. V. Pimerova, E. V. Voronina, *J. Pharm. Chem.*, **35**, 18 (2001)
35. R. H. Wiley, P. Wiley, *Pyrazolones, Pyrazolidones and Derivatives* (John Wiley and Sons New York) p. 102, 1964
36. V. Michon, C. H. D. Penhoat, F. Tombret, J. M. Gillardin, F. Lepagez, L. Berthon, *Eur. J. Med. Chem.*, **30**, 147 (1995)
37. S. L. Janus, A. Z. Magdif, B. P. Erik, N. Claus, *Monatsh. Chem.*, **130**, 1167 (1999)
38. K. L. Kees, J. J. J. Fitzgerald, K. E. Steiner, J. F. Mattes, B. Mihan, T. Tosi, D. Mondoro, M. L. McCaleb, *J. Med. Chem.*, **39**, 3920 (1996)
39. Shamsuzzaman, M. Aslam, A. Salim, *J. Chem. Res. (S)*, 824 (1998)
40. G. Socrates, *Infrared Characteristic Group Frequencies* (John Wiley and Sons) p. 102, 1980
41. M. Himly, B. Jahn-Schmid, K. Pittertschatscher, B. Bohle, K. Grubmayr, F. Ferreira, H. Ebner, C. Ebner, *J. Allergy Clin. Immunol.*, **111**, 882 (2003)
42. T. Watanabe, S. Yuki, M. Egawa, H. Nishi, *J. Pharmacol. Exp. Ther.*, **268**, 1597 (1994)
43. T. W. Wu, L. H. Zeng, J. Wu, K. P. Fung, *Life Sci.*, **71**, 2249 (2002)
44. M. A. Al-Haiza, S. A. El-Assiery, G. H. Sayed, *Acta Pharm.*, **51**, 251 (2001)
45. D. Castagnolo, F. Manetti, M. Radi, B. Bechi, M. Pagano, A. De Logu, R. Meleddu, M. Saddi, M. Botta, *Bioorg. Med. Chem.*, **17**, 5716 (2009)
46. E. A. M. Badawey, I. M. El-Ashmawey, *Eur. J. Med. Chem.*, **33**, 349 (1998)
47. F. A. Pasha, M. Muddassar, M. M. Neaz, S. J. Cho, *J. Mol. Graph. Model.*, **28**, 54 (2009)
48. C. E. Rosiere, M. I. Grossman, *Science*, **113**, 651 (1951)
49. D. M. Bailey, P. E. Hansen, A. G. Hlavac, E. R. Baizman, J. Pearl, A. F. Defelice, M. E. Feigenson, *J. Med. Chem.*, **28**, 256 (1985)
50. P. M. S. Chauhan, S. Singh, R. K. Chatterjee, *Indian J. Chem.*, **32B**, 858 (1993)

51. B. Stanovnik, J. Svete, *Sci. Synth.*, **12**, 220 (2002)

52. A. Tong, Y. Akama, S. Tanaka, *J. Chromatogr.*, **478**, 408 (1989)

53. H. Samelson, A. Lempicki, *J. Chem. Phys.* **39**, 110 (1963)

54. R. G. Charles, E. P. Riedel, *J. Inorg. Nucl. Chem.*, **28**, 3005 (1966)

55. C. W. Shoppe, R. H. Jenkins, G. H. R. Summers, *J. Chem. Soc.*, 1657 (1958)

56. R. M. Dodson, B. Riegel, *J. Org. Chem.*, **13**, 424 (1948)

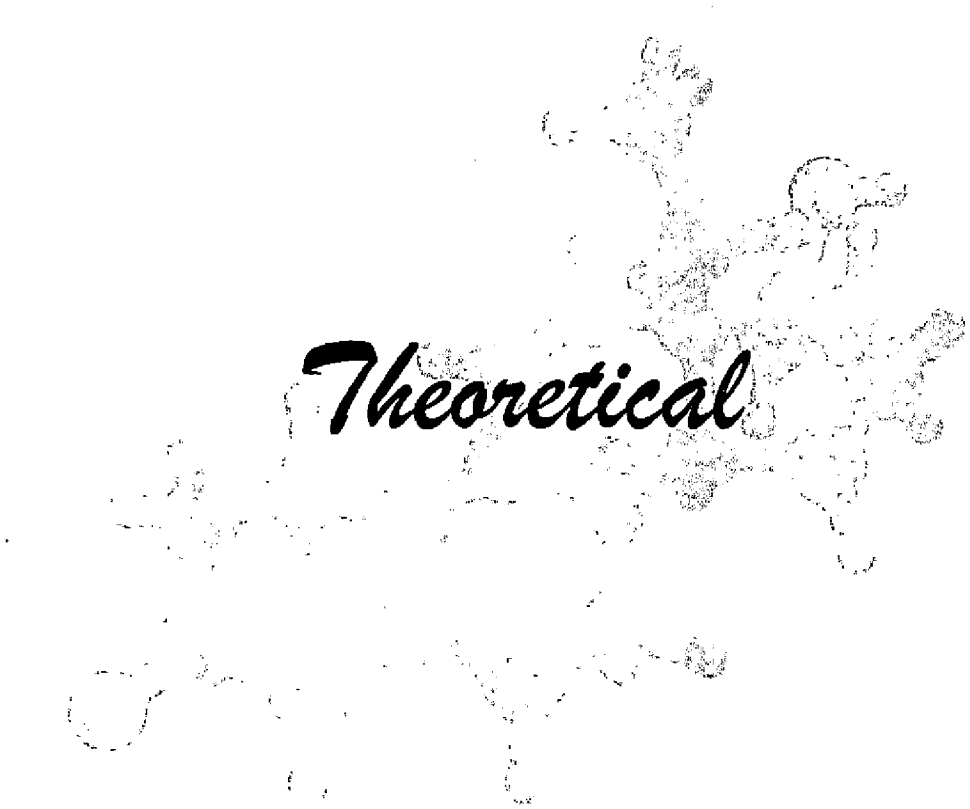
57. A. Windus, O. Dalmer, *Chem. Ber.*, **52**, 162 (1919)

58. S. Bondock, A. E. Tarhoni, A. A. Fadda, *Arkivoc*, **9**, 113 (2006)

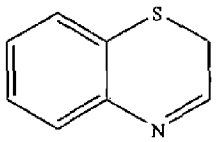
Chapter-4

Synthesis of steroidal benzothioazines and thiazoles

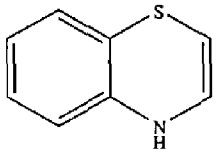
Theoretical



Benzothiazine is a heterocyclic compound consisting of a benzene ring attached to the 6-membered heterocycle thiazine. The molecular formula is C_8H_7NS. The name is applied to both the 2H isomer (1) and 4H isomer (2) which differ by the location of the double bonds. Benzothiazines were first reported in the 1960s. Subsequently, their preparation and intensive biological studies have been reported. In recent years benzothiazines have been of enormous interest to synthetic chemists. The enantioselective synthesis of such benzothiazines has been developed and formulated and transformations of these compounds were designed to target chiral, non-racemic building blocks as well as natural products.

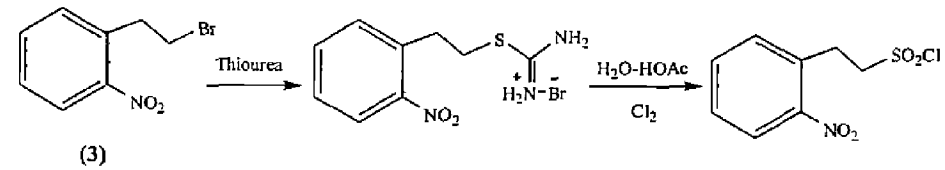


(1)

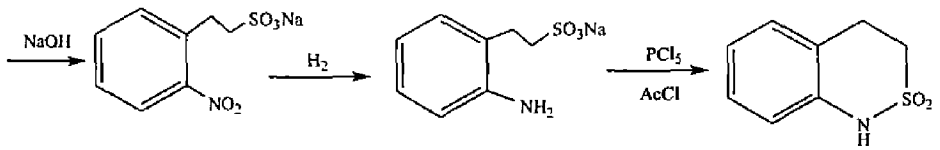


(2)

Loev and Kormendy[1] in 1965 reported the first procedure for the synthesis of sulfostyril (2, 1-benzothiazine 2, 2-dioxide) (4) during which 2-nitrophenethyl bromide (3) was converted in two steps to sulfonyl chloride. Alkaline hydrolysis gave the sodium sulfonate which was catalytically reduced to the amine. The amine upon trituration with PCl_5 yielded desired product sulfostyril (4).

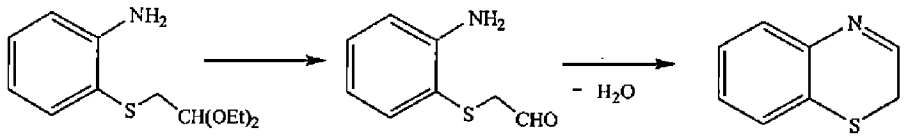


(3)



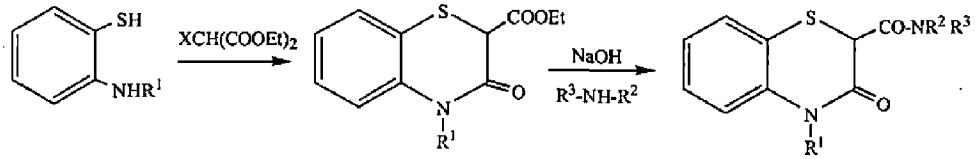
(4)

Prota and co-workers[2] reported the hydrolysis of (1-*o*-aminophenylthio-2, 2-diethoxy) ethane (5) resulted in the formation of (2-*o*-aminophenylthio)acetaldehyde (6) which after condensation formed ring in the form of 2H-1, 4-benzothiazine (7).



(5) (6) (7)

Corona *et al.*[3] reported that N-substituted aminothiophenols (**8 a-c**) reacted with haloester and gave 3-oxo-3, 4-dihydro-2H-benzothiazine-2-carboxylic acid ethyl ester derivatives (**9 a-c**) which upon reaction with different amines provided 2H-1, 4-benzo-thiazine-3, 4-dihydro-3-oxo-2-carboxamides (**10 a-h**) in good yields.



(8)

R^1
(a) H
(b) CH_3
(c) C_2H_5

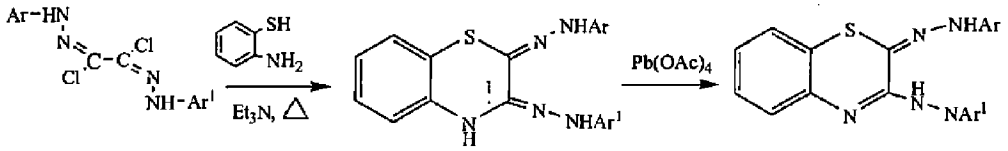
(9)

R^1
(a) H
(b) CH_3
(c) C_2H_5

(10)

R^1	**R^2**	**R^3**
(a) H	H	C_6H_4Cl
(b) CH_3	CH_3	CH_3
(c) C_2H_5	C_2H_5	$C_6H_4OCH_3$
(d) H	H	$C_6H_4OCH_3$
(e) H	C_2H_5	$C_6H_3O_2CH_2$
(f) H	CH_3	C_6H_5
(g) C_2H_5	H	C_6H_4Cl
(h) C_2H_5	H	$C_6H_4OCH_3$

Farag and co-workers[4] reported that the aryl hydrazonoyl dichlorides (**11 a-c**) on reaction with 2-aminothiophenol in refluxing ethanol in the presence of triethylamine afforded 2, 3-bis(arylhydrazono)-2, 3-dihydro-4H-1, 4-benzothiazines (**12 a-c**) in good yields which on treatment with lead tetracetate in acetic acid at room temperature provided respective oxidation products (**13 a-c**).



(11)

Ar	**Ar^1**
(a) C_6H_5	C_6H_5
(b) ClC_6H_4	ClC_6H_4
(c) $Cl_2C_6H_3$	$Cl_2C_6H_3$

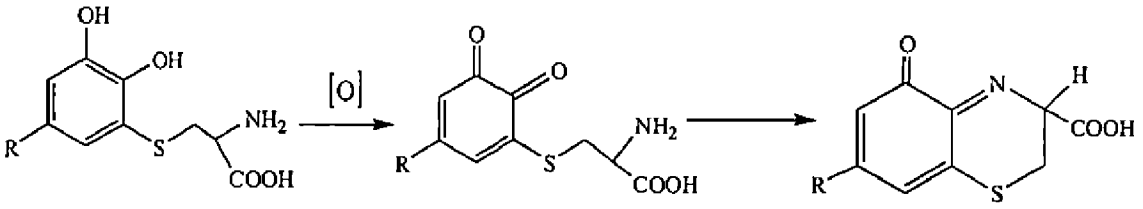
(12)

Ar	**Ar^1**
(a) C_6H_5	C_6H_5
(b) ClC_6H_4	ClC_6H_4
(c) $Cl_2C_6H_3$	$Cl_2C_6H_3$

(13)

Ar	**Ar^1**
(a) C_6H_5	C_6H_5
(b) ClC_6H_4	ClC_6H_4
(c) $Cl_2C_6H_3$	$Cl_2C_6H_3$

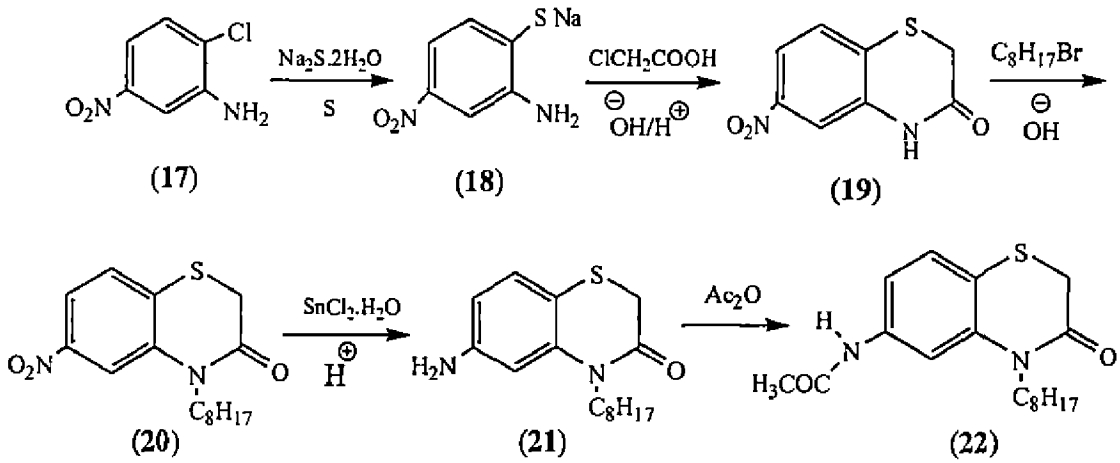
Napolitano and co-workers[5] reported that the oxidation of cysteinylcatechols (**14 a-d**) gave cysteinyldopaquinones (**15 a-d**) which after ring closure of the cysteine side chain under reflux conditions yielded 2H-benzothiazine derivatives (**16 a-d**).



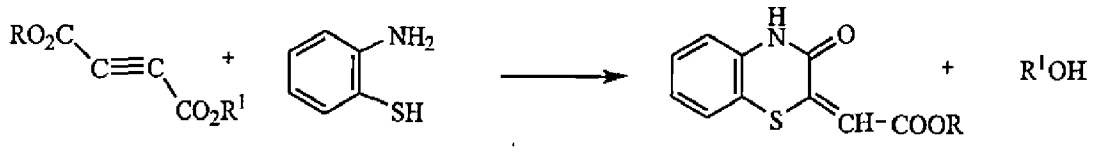
(**14**) (**15**) (**16**)

R	R	R
(a) CH_2-CH(NH_2)-COOH	(a) CH_2-CH(NH_2)-COOH	(a) CH_2-CH(NH_2)-COOH
(b) CH_3	(b) CH_3	(b) CH_3
(c) OCH_3	(c) OCH_3	(c) OCH_3
(d) OC_2H_5	(d) OC_2H_5	(d) OC_2H_5

Guarda *et al.*[6] reported that the treatment of 2-chloro-5-nitroaniline (**17**) with sodium sulfide and sulfur gave 2-amino-4-nitrobenzenethiol sodium salt (**18**), which was cyclized to 2H-1, 4-benzothiazin-3-one (**19**), with chloroacetic acid. N-alkylation with octyl bromide and KOH in methanol afforded 4-octyl-6-nitro-2H-1, 4-benzothiazin-3-one (**20**). Reduction of the nitro group of **20** by $SnCl_2$ in acidic medium gave 6-amino-4-octyl-2H-1, 4-benzothiazin-3-one (**21**). The N-acetylation of **21** resulted in acetylated product, acetylamino-4-octyl-2H-1, 4-benzothiazin-3-one (**22**).



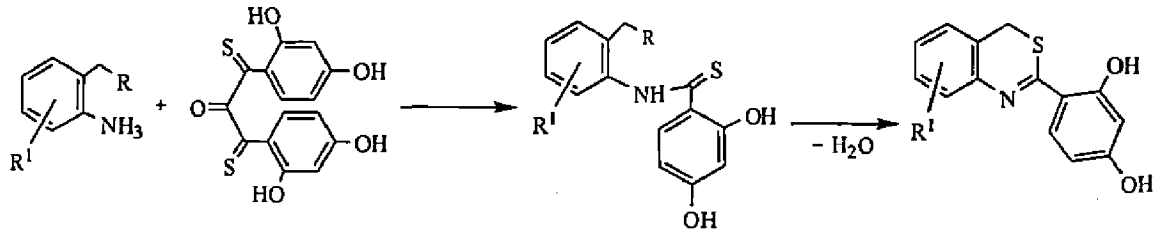
Esmaili and co-workers[7] reported that a magnetically stirred solution of 2-aminothiophenol in 40% ethyl acetate/hexane (5 mL) was allowed to react with the solution of dimethyl acetylenedicarboxylate (**23 a, b**) in 40% ethyl acetate/hexane (2 mL) that resulted in the formation of 3, 4-dihydro-3-oxo-2H-benzo-1, 4-thiazine derivatives (**24 a, b**).



(23) (24)

R	R^1	R	R^1
(a) *iso*-Pr	*iso*-Pr	(a) *iso*-Pr	*iso*-Pr
(b) *tert*-Bu	*tert*-Bu	(b) *tert*-Bu	*tert*-Bu

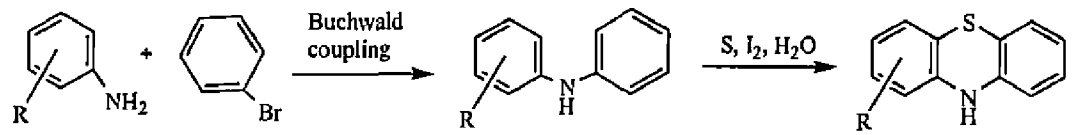
Joanna Matysiak[8] reported that reaction between substituted anilines (**25 a-d**) and sulfinyl bis (2, 4-dihydroxythiobenzoyl (STB) resulted in the formation of 2, 4-dihydroxy-N-(substituted phenyl) thiobenzamide derivatives (**26 a-d**) which in turn underwent intramolecular cyclization and gave 2-(2, 4-dihydroxy substituted phenyl)-4H-3, 1-benzo thiazines (**27 a-d**).



(25) (26) (27)

R	R^1	R	R^1	R^1
(a) OH	OH	(a) OH	OH	(a) OH
(b) NH_2	OCH_3	(b) NH_2	OCH_3	(b) OCH_3
(c) $NHCH_3$	NH_2	(c) $NHCH_3$	NH_2	(c) NH_2
(d) $N(CH_3)_2$	$NHCH_3$	(d) $N(CH_3)_2$	$NHCH_3$	(d) $NHCH_3$

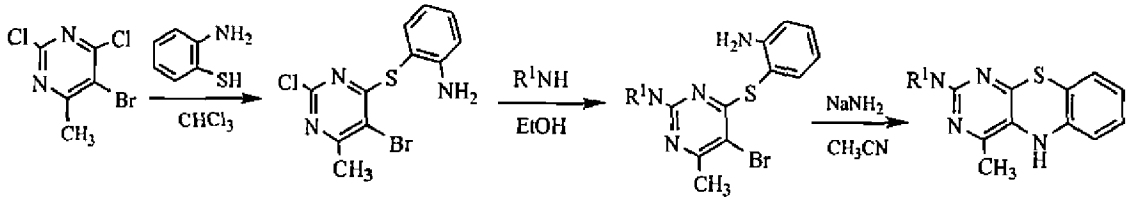
Madrid *et al.*[9] reported the synthesis of diphenylamine derivatives (**29 a-d**) by the coupling of substituted aniline (**28 a-d**) with bromobenzene using Buchwald palladium coupling reaction. The compounds **29** (**a-d**) were then cyclized through reaction with sulfur and catalytic iodide under microwave irradiation and yielded 10H-phenothiazines (**30 a-d**).



(28) (29) (30)

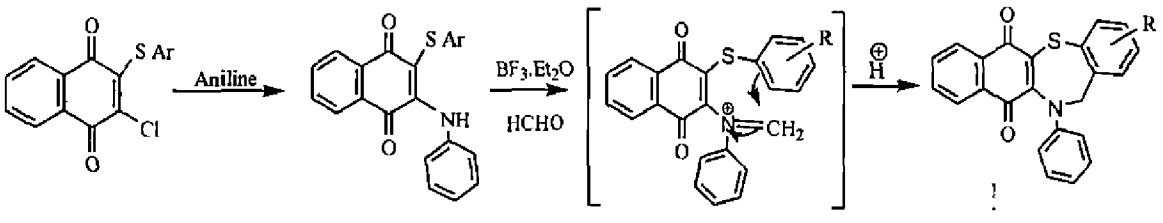
R	R	R
(a) COOEt	(a) COOEt	(a) COOEt
(b) CN	(b) CN	(b) CN
(c) CF_3	(c) CF_3	(c) CF_3
(d) CH_3	(d) CH_3	(d) CH_3

Bakavoli and co-workers[10] reported the reaction of 5-bromo-2, 4-dichloro-6-methyl pyrimidine (**31**) with 2-aminothiophenol which gave 2-(5-bromo-2-chloro-6-methyl pyrimidin-4-ylthio) benzenamine (**32**). The compound 32 reacted with secondary amines and yielded 2-(5-bromo-6-methyl-2-substituted-aminopyrimidin-4-ylthio) benzene amines (**33 a-h**) which on further reaction with sodamide in acetonitrile furnished pyrimido [4, 5 - *b*] [1, 4] benzothiazines (**34 a-h**) in good yield.



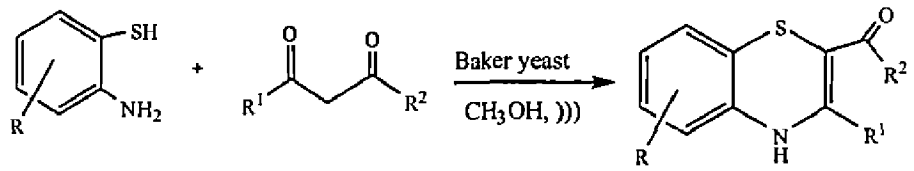
R^1	**R^1**
(a) Pyrrolidine	(a) Pyrrolidine
(b) Piperidine	(b) Piperidine
(c) Methylpiperidine	(c) Methylpiperidine
(d)Methyl-6H-pyrimidine	(d) Methyl-6H-pyrimidine
(e) Ethyl-6H-pyrimidine	(e) Ethyl-6H-pyrimidine
(f) Morpholine	(f) Morpholine
(g) Piperidin-4-ol	(g) Piperidin-4-ol
(h) 1-Phenyl-piperazine	(h) 1-Phenyl-piperazine

Tandon and co-workers[11] reported the reaction of 2-chloro-3-aryl sulfanyl-[1, 4] naphthoquinones (**35 a-c**) with aniline that provided 2-phenylamino-3-aryl sulfanyl-[1, 4] naphthoquinones (**36 a-c**) which in turn reacted with *p*-formaldehyde in the presence of tetrafluoroborate to yield quaternary intermediate that underwent intramolecular cyclization and gave dihydrobenzo [*f*] naphtha [2, 3 - *b*][1, 4] thiazepine-6, 11-dione (**37 a-c**).



Ar	**Ar**	**R**
(a) C_6H_5	(a) C_6H_5	(a) H
(b) 3-$OMeC_6H_4$	(b) 3-$OMeC_6H_4$	(b) 3-OMe
(c) Naphthyl	(c) Naphthyl	(c) C_4H_{12}

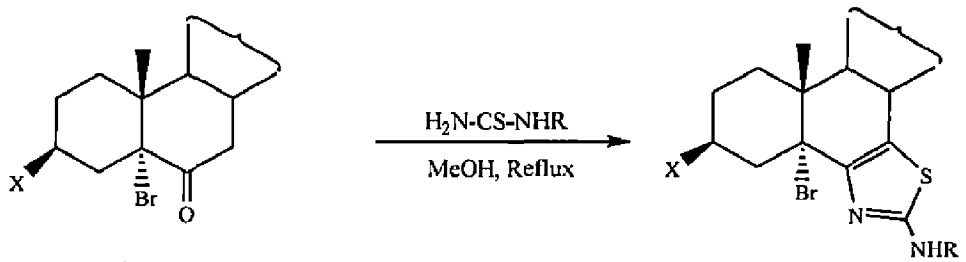
Pratap and co-workers[12] reported the synthesis of 1, 4-benzothiazine derivatives (38 a-j) by the condensation of 2-aminobenzenethiols and 1, 3-dicarbonyl compounds using biocatalyst, baker's yeast under ultrasonic conditions.



(38)

R	R^1	R^2	R	R^1	R^2
(a) H	(a) CH_3	CH_3	(a) H	CH_3	CH_3
(b) CH_3	(b) CH_3	OC_2H_5	(b) H	CH_3	OC_2H_5
(c) Cl	(c) CH_3	Ph	(c) H	CH_3	Ph
	(d) CH_3	CH_3	(d) CH_3	CH_3	CH_3
	(e) CH_3	OC_2H_5	(e) CH_3	CH_3	OC_2H_5
	(f) CH_3	Ph	(f) CH_3	CH_3	Ph
	(g) CH_3	CH_3	(g) Cl	CH_3	CH_3
	(h) CH_3	OC_2H_5	(h) Cl	CH_3	OC_2H_5
	(i) CH_3	Ph	(i) Cl	CH_3	Ph
	(j) Ph	Ph	(j) H	Ph	Ph

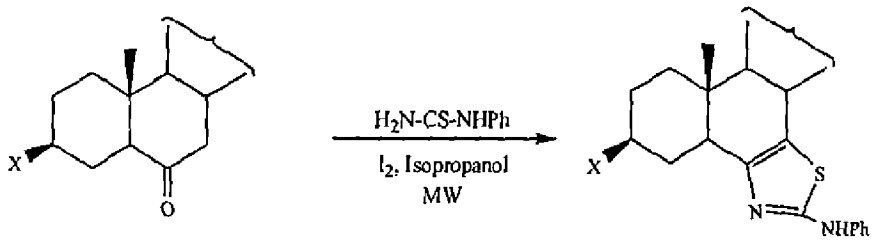
Alam *et al.*[13] reported the synthesis of amino and phenyl amino derivatives of 5α-cholest-6-eno [6, 7 - *d*] thiazole (**40 a-f**) by reacting the lachrymatory 5-bromosteroidal ketones (**39 a-c**) with thiourea or phenylthiourea in methanol under reflux conditions.



(39) (40)

X	X	R
(a) OAc	(a) OAc	H
(b) C_2H_5COO	(b) C_2H_5COO	H
(c) H	(c) H	H
	(d) OAc	Ph
	(e) C_2H_5COO	Ph
	(f) H	Ph

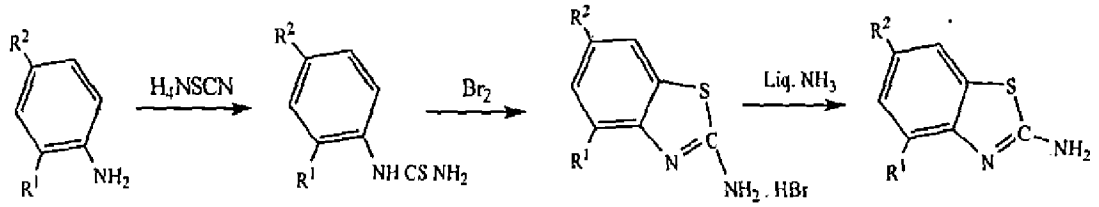
Mushfiq *et al.*[14] modified the procedure reported by Alam *et al.*[13] and reported the one pot synthesis of 2′-amino-5α-cholest-6-eno [6, 7 - *d*] thiazole derivatives (**42 a-c**) by the reaction of steroidal ketones (**41 a-c**) with iodine and phenylthiourea under microwave conditions.



(41) (42)

X	X
(a) H	(a) H
(b) OAc	(b) OAc
(c) OPr	(c) OPr

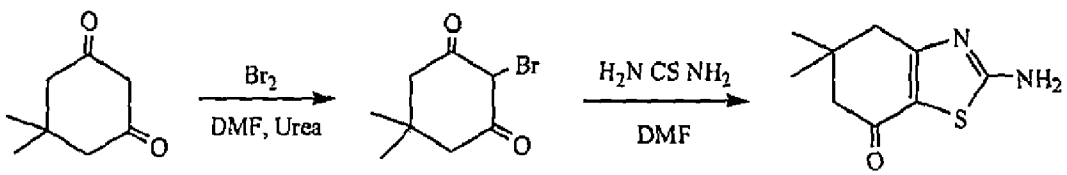
Kumar and co-workers[15] reported the reaction of 2, 4-substituted aniline derivatives (43 a-d) with ammonium thiocyanate that yielded 2, 4-substituted phenylthiourea derivatives (44 a-d) which in turn reacted with bromine and gave substituted benzothiazol-2-ylamine bromate derivatives (45 a-d) which later upon reacted with liquid ammonia yielded benzothiazole derivatives (46 a-d).



(43) (44) (45) (46)

R¹	R²	R¹	R²	R¹	R²	R¹	R²
(a) CH_3	H	(a) CH_3	H	(a) CH_3	CH_3	(a) CH_3	H
(b) CF_3	H	(b) CF_3	H	(b) CF_3	H	(b) CF_3	H
(c) OCH_3	H	(c) OCH_3	H	(c) OCH_3	H	(c) OCH_3	H
(d) H	Br	(d) H	Br	(d) H	Br	(d) H	Br

Zav'yalov and co-workers[16] reported that 5, 5-dimethyl-cyclohexane-1, 3-dione (47) underwent reaction with bromine to provide 2-bromo-5, 5-dimethyl-cyclohexane-1, 3-dione (48) which in turn reacted with thiourea to yield 2-amino-5, 5-dimethyl-5, 6-dihydro-4H-benzo-1, 3-thiazol-7-one (49).



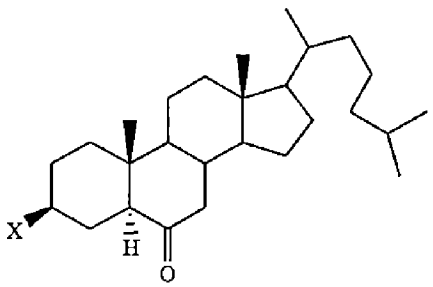
(47) (48) (49)

Discussion

Nitrogen containing steroids have the ability to regulate a variety of biological processes and thus are potential drug candidates for the treatment of a large number of diseases including breast cancer, prostate cancer, leukaemia, autoimmune diseases and osteoporosis.[17-21] So is the case with the nitrogen containing derivative, benzothiazines in which the presence of a fold along the nitrogen-sulfur axis is one of the features responsible to impart their biological activity[22], hence they show broad spectrum of biological activities such as antagonists, anticancer, vasorelaxant, antidiabetic, antihypertensive and antimicrobial.[23-28]

Thiazole derivatives have also attracted continuing interest over the years because of their varied biological activities. They have been reported as antiallergic, antihypertensive, anti-inflammatory, antischizophrenic, antibacterial, anti-HIV, hypnotic, selective COX-2 inhibitors, fibrinogen receptor antagonists with antithrombotic activity and inhibitors of bacterial DNA gyrase B.[29-38] The substituted thiazoles have a number of other characteristic pharmacological features such as relative stability and ease of starting materials built in biocidal unit, enhanced lipid solubility with hydrophilicity and easy metabolism of compounds.[39]

The biological importance of these steroidal benzothiazines[25-30] and steroidal thiazoles[29-37] encouraged us to undertake the synthesis of new steroidal benzothiazines and aminothiazoles. The substrates selected for synthesizing these steroidal derivatives include 5α-cholestan-6-one[40] (**50**), 3β-acetoxy-5α-cholestan-6-one[41] (**51**) and 3β-chloro-5α-cholestan-6-one[42] (**52**). The products obtained have been characterized on the basis of spectral (IR, ^{1}H NMR, ^{13}C NMR, MS) and elemental analyses.

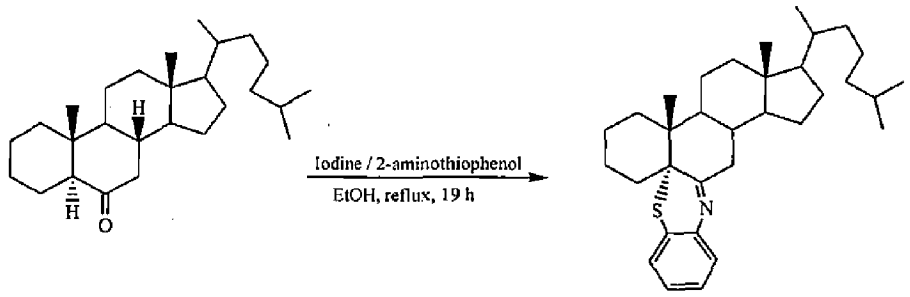


X

(**50**) H
(**51**) OAc
(**52**) Cl

Reaction of 5α-cholestan-6-one (50) with iodine and 2-aminothiophenol.

The 5α-cholestan-6-one (**50**) (1 mmol) in absolute ethanol (10 mL) was allowed to react with 2-aminothiophenol (1 mmol) and iodine (2 mmol). After completion of the reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to give compound **53**, m.p 152 °C.



(50) **(53)**

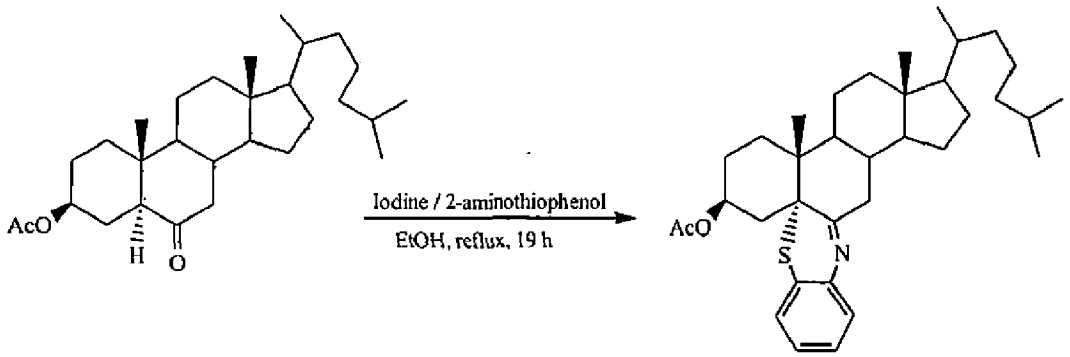
Characterization of the compound, m.p. 152 °C as 5α-cholestano [5, 6 - *b*] benzothiazine (53)

The compound **53** was correctly analyzed for the molecular formula $C_{33}H_{48}NS$. Its IR spectrum showed a band at 1628 cm^{-1} assigned to C=N group while as the bands at 3062 and 1600 cm^{-1} confirmed the presence of an aromatic moiety. The bands at 711 and 1385 cm^{-1} were attributed to C-S and C-N groups, respectively. These values supported the presence of benzothiazine moiety[43] in the product molecule. The structure **53** was well supported by its ^1H NMR spectrum which displayed a multiplet at δ 6.43-6.24 integrating for four protons, indicating the presence of an aromatic ring. A two-proton doublet appeared at δ 2.05 (J = 8.0 Hz) for C_4-H_2 and another doublet at δ 2.04 (J = 4.4 Hz) for C_7-H_2. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.17, 0.97, 0.80 and 0.75. The ^{13}C NMR spectrum of compound **53** displayed characteristic signals at δ 163 showing the presence of C=N while as the signals at 149, 125, 124, 122.8, 122 and 120 confirmed the presence of an aromatic ring. Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **53** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 491) was found.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 4.1**), this compound can be best characterized as 5α-cholestano [5, 6 - *b*] benzothiazine **53**.

Reaction of 3β-acetoxy-5α-cholestan-6-one (51) with iodine and 2-aminothiophenol.

The 3β-acetoxy-5α-cholestan-6-one (**51**) (1 mmol) in absolute ethanol (10 mL) was allowed to react with 2-aminothiophenol (1 mmol) and iodine (2 mmol). After completion of the reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to give compound **54**, m.p 163 °C.



(51) **(54)**

Characterization of the compound, m.p. 163 °C as 3β-acetoxy-5α-cholestano [5, 6 - *b*] benzothiazine (54)

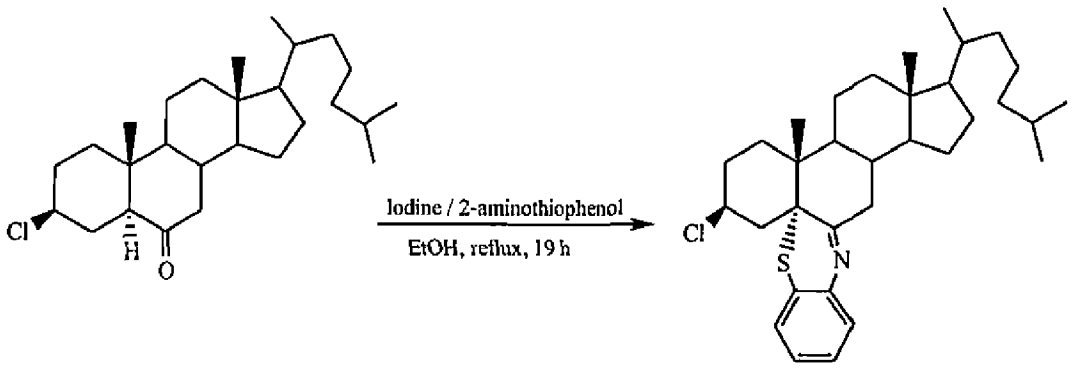
The elemental analysis of the compound corresponded to the molecular formula $C_{35}H_{51}NO_2S$. Its IR spectrum showed a band at 1650 cm^{-1} which could be assigned to C=N group while as the bands at 3060 and 1603 cm^{-1} confirmed the presence of an aromatic moiety. The IR spectrum of the compound **54** exhibited strong absorption bands at 1714 and 1206 cm^{-1} indicating the presence of an acetoxy group while as the bands at 750 and 1388 cm^{-1} were attributed to C-S and C-N groups, respectively. These values suggested the presence of benzothiazine moiety[43] in the product molecule. The structure **54** was well supported by its ^1H NMR spectrum which displayed a multiplet at δ 6.33-6.28 integrating for four protons, indicating the presence of an aromatic ring. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. The three acetoxy group protons appeared at δ 2.03 as a sharp singlet. A doublet for two protons appeared at δ 1.8 (J = 8.0 Hz) for C_4-H_2 and another doublet integrating for two protons at δ 1.9 (J = 5.2 Hz) for C_7-H_2. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound **54** displayed characteristic signals at δ 174 and 163 showing the presence of *C*=O and *C*=N, respectively while as the signals at 148, 129, 128, 126, 124 and 122 confirmed the presence of an aromatic carbons. The signal at δ 73 was assigned to the C_3 of the steroidal molecule

with acetoxy group attached to it. Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **54** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 549) was observed.

On the basis of above studies and its analogy with earlier compound **53**, this compound can be best characterized as 3*β*-acetoxy-5*α*-cholestano [5, 6 - *b*] benzothiazine **54**.

Reaction of 3*β*-chloro-5*α*-cholestan-6-one (52) with iodine and 2-aminothiophenol.

The 3*β*-chloro-5*α*-cholestan-6-one (**52**) (1 mmol) in absolute ethanol (10 mL) was allowed to react with 2-aminothiophenol (1 mmol) and iodine (2 mmol). After completion of the reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvents gave an oil which was crystallized from methanol to give compound **55**, m.p 146 °C.



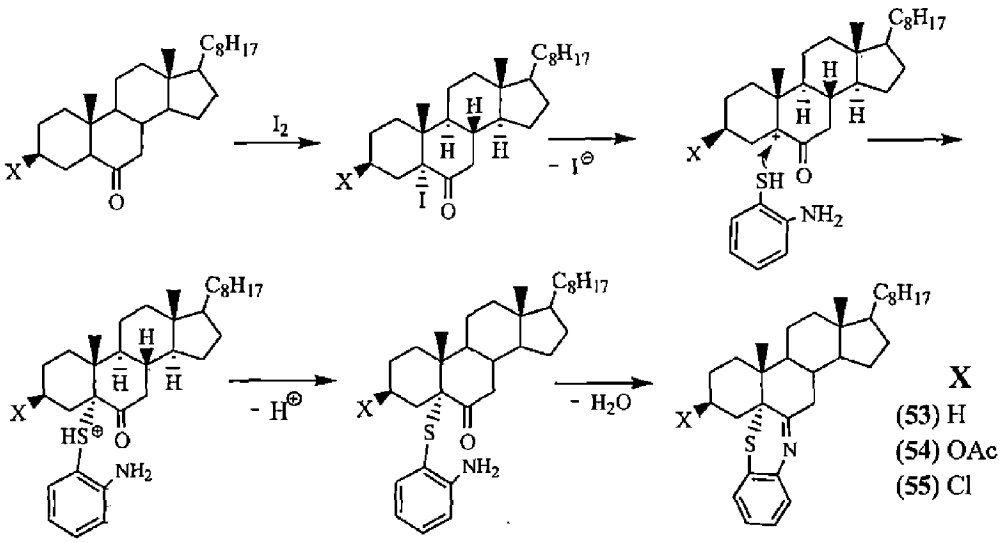
(52) **(55)**

Characterization of the compound, m.p. 146 °C as 3*β*-chloro-5*α*-cholestano [5, 6 - *b*] benzothiazine (55)

The compound **55** was correctly analyzed for the molecular formula $C_{33}H_{48}ClNS$ (Beilstein positive). Its IR spectrum showed a band at 1626 cm^{-1} which could be assigned to C=N group while as the bands at 3058 and 1598 cm^{-1} confirmed the presence of an aromatic moiety and the bands at 710, 1380 and 740 cm^{-1} were attributed to C-S, C-N and C-Cl groups, respectively. These values supported the presence of benzothiazine moiety[43] in the product molecule. The structure **55** was well supported by its ^1H NMR spectrum which displayed a multiplet at δ 6.43-6.24 for four protons, indicating the presence of an aromatic ring. A broad multiplet ($W_{1/2}$ = 17 Hz, axial) for one proton was observed at δ 3.5 which could be assigned to $C_3\alpha$-*H*. A doublet for two protons appeared at δ 2.07 (J = 8.0 Hz) for C_4-H_2 and another doublet at δ 1.87 (J = 4.8 Hz) for two C_7-methyl protons. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.80 and 0.75.

The ^{13}C NMR spectrum of compound **55** displayed a characteristic signal at δ 164 showing the presence of *C*=N while as the signals at 146, 127, 126,.125, 123 and 122 confirmed the presence of an aromatic moiety. The signal at δ 59 was assigned to the C_3 of the steroidal molecule with chlorine attached to it. Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **55** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 525/527) was found.

Formation of steroidal benzothiazines (**53-55**) under the condition case may be shown according to the proposed mechanism (**Scheme 4.1**). The mechanism for the formation of these benzothiazines involves formation of 5*α*-iodocholest-6-one *in situ* as an intermediate, which on further reaction with 2-aminothiophenol undergoes SN^1 reaction at C_5 and condensation at C_6 resulting in cyclization that leads to the formation of corresponding product. The remarkable feature of the reaction is the formation of 5*α*-iodoketone *in situ* as an intermediate which might be obtained separately by the reaction of ketones with iodine.



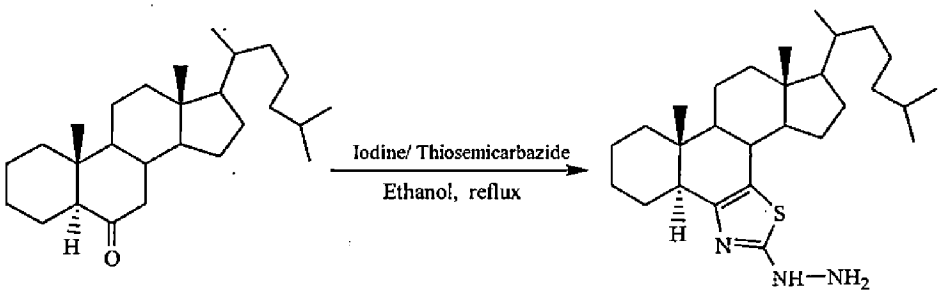
Scheme 4.1. Mechanism for the formation of steroidal benzothiazine derivatives (**53-55**)

Work published;

Anticancer and antimicrobial evaluation of newly synthesized steroidal 5, 6 fused benzothiazines. Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Arabian Journal of Chemistry*, http://dx.doi.org/10.1016/j.arabjc.2013.06.027 (in press)

Reaction of 5α-cholestan-6-one (50) with iodine and thiosemicarbazide.

The 5α-cholestan-6-one (**50**) (1 mmol) in absolute ethanol (15 mL) was allowed to react with thiosemicarbazide (1 mmol) and iodine (2 mmol). After completion of the reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvent gave an oil which was crystallized from methanol to afford compound **56**, m.p 129 °C.



(50) **(56)**

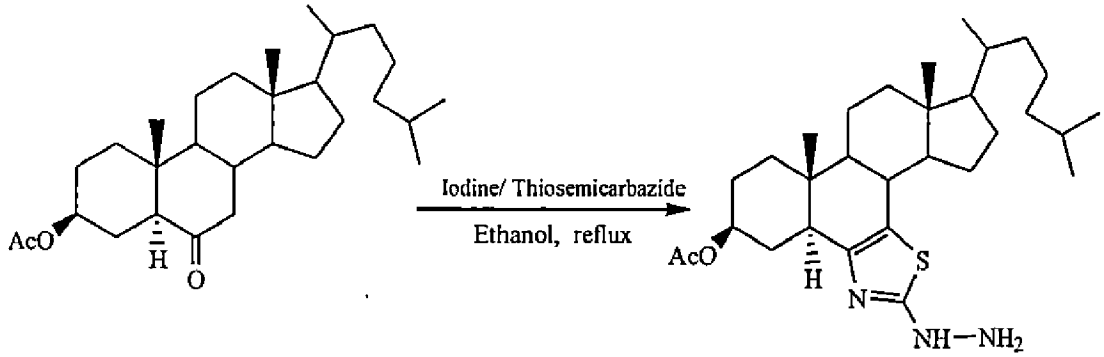
Characterization of the compound, m.p. 129 °C as 2'-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (56)

The compound **56** was correctly analyzed for the molecular formula $C_{28}H_{47}N_3S$. Its IR spectrum showed bands at 3376 and 3328 cm^{-1} which could be assigned to NH and NH_2 groups, respectively while as the bands at 1617, 1557, 1328 and 634 cm^{-1} were attributed to C=C, C=N, C-N and C-S groups, respectively. These values suggested the presence of aminothiazole moiety[43] in the product molecule. The structure **56** was well supported by its ^1H NMR spectrum which displayed a broad singlet integrating for two protons at δ 6.2 (exchangeable with D_2O) depicted the presence of NH_2 while as the singlet integrating for one proton at δ 3.8 (exchangeable with D_2O) showed the presence of N*H*. A double doublet appeared at δ 2.74 (J = 16.9 and 5.5 Hz) depicting the presence of $C_5\alpha$-*H*. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound **56** displayed characteristic signal at δ 163 showing the presence of *C*=N while as the signals at δ 130 and 120 assigned to C_6 and C_7, respectively. Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **56** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 457) was found.

On the basis of foregoing discussion and the mechanism proposed (**Scheme 4.2 a, b**), this compound can be best characterized as, 2'-hydrazinocholest-6-eno [4, 5 - *d*] thiazole **56**.

Reaction of 3β-acetoxy-5α-cholestan-6-one (51) with iodine and thiosemicarbazide.

The 3β-acetoxy-5α-cholestan-6-one (51) (1 mmol) in absolute ethanol (15 mL) was allowed to react with thiosemicarbazide (1 mmol) and iodine (2 mmol). After completion of reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvent gave an oil which was crystallized from methanol to give compound 57, m.p 136 °C.



(51) (57)

Characterization of the compound, m.p. 136 °C as 3β-acetoxy-2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (57)

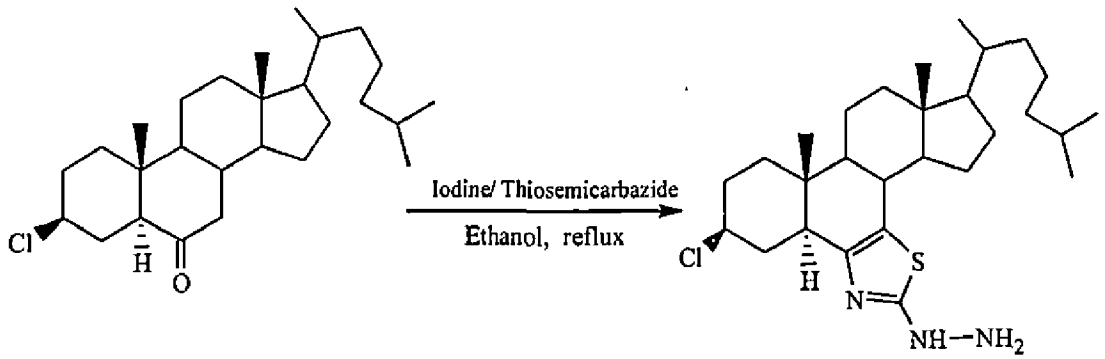
The elemental analysis of compound 57 corresponded to the molecular formula $C_{30}H_{49}N_3O_2S$. Its IR spectrum showed bands at 3395 and 3310 cm^{-1} which could be assigned to NH and NH_2 groups, respectively. The IR spectrum of the compound exhibited strong absorption bands at 1730 and 1210 cm^{-1} indicating the presence of acetate group while as the bands at 1625, 1555, 1320 and 645 cm^{-1} were attributed to C=C, C=N, C-N and C-S groups, respectively. These IR values supported the presence of aminothiazole moiety[43] in the product molecule. The structure 57 was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 6.8 (exchangeable with D_2O) indicating the presence of N*H*$_2$ while as a singlet integrating for one proton at δ 4.4 (exchangeable with D_2O) showed the presence of N*H*. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 4.7 which could be assigned to $C_3\alpha$-*H*. The three acetoxy group protons appeared at δ 2.03 as a sharp singlet. A double doublet for one proton appeared at δ 2.7 (J = 15 and 5 Hz) for $C_5\alpha$-*H*. Other prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound 57 displayed characteristic signals at δ 171.2, 163 showing the presence of *C*=O and *C*=N while as the signals at δ 132, 120 and 70.2 were assigned to C_6, C_7 and C_3, respectively. Remaining carbon atoms were seen in accordance to the cholestane series. The

structure of compound **57** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 515) was found.

On the basis of above studies and its analogy with earlier compound **56**, this compound can be best characterized as 3*β*-acetoxy-2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (**57**).

Reaction of 3*β*-chloro-5*α*-cholestan-6-one (52) with iodine and thiosemicarbazide.

The 3*β*-chloro-5*α*-cholestan-6-one (**52**) (1 mmol) in absolute ethanol (15 mL) was allowed to react with thiosemicarbazide (1 mmol) and iodine (2 mmol). After completion of reaction, the reaction mixture was taken in diethyl ether, diluted with $Na_2S_2O_7$ solution, subsequently washed with water and dried over anhydrous sodium sulfate. Removal of the solvent gave an oil which was crystallized from methanol to give compound **58**, m.p 143 °C.



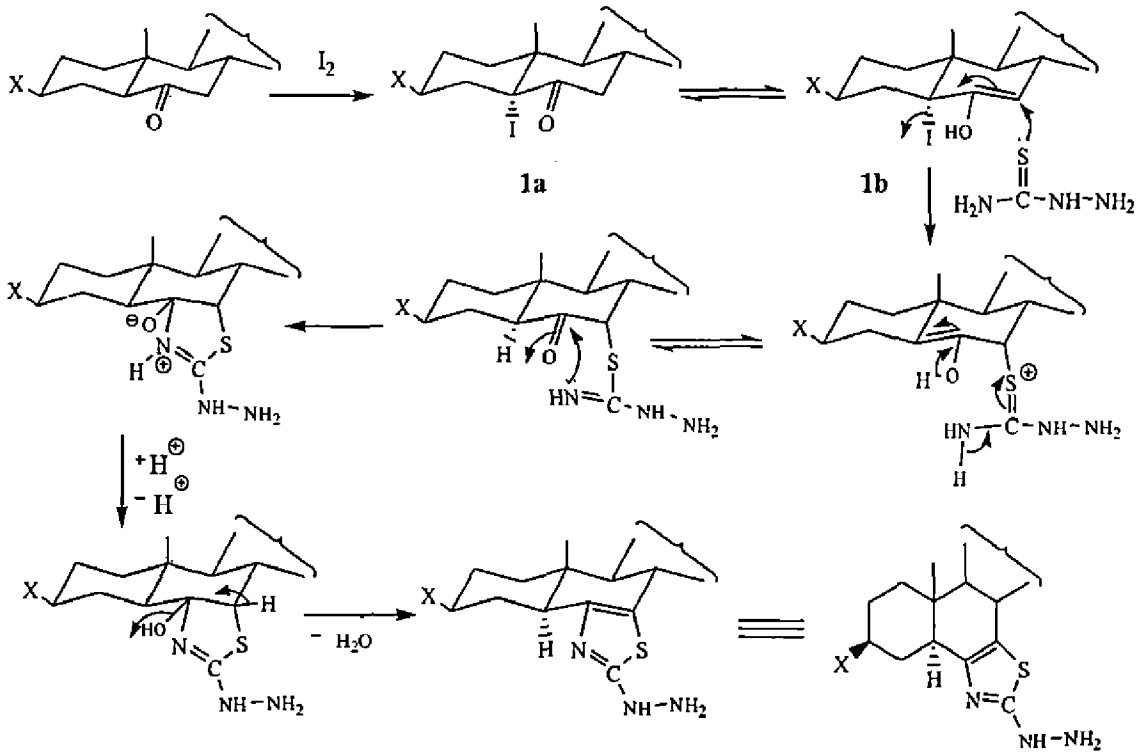
(**52**) (**58**)

Characterization of the compound, m.p. 143 °C as 3*β*-chloro-2′-hydrazinocholest-6-eno [4, 5 - *d*] thiazole (58)

The compound **58** was correctly analyzed for the molecular formula $C_{28}H_{46}ClN_3S$ (Beilstein positive). Its IR spectrum showed bands at 3370 and 3320 cm^{-1} which could be assigned to NH and NH_2 groups, respectively. The IR spectrum of the compound **58** exhibited absorption bands at 1622, 1560, 1323, 745 and 635 cm^{-1} which were attributed to C=C, C=N, C-N, C-Cl and C-S groups, respectively. These IR values depicted the presence of aminothiazole moiety[43] in the product molecule. The structure **58** was well supported by its ^1H NMR spectrum which displayed broad singlet integrating for two protons at δ 6.63 (exchangeable with D_2O) indicating the presence of NH_2 while as a singlet integrating for one proton at δ 4.45 (exchangeable with D_2O) showed the presence of N*H*. A broad multiplet ($W_{1/2}$ = 15 Hz, axial) for one proton was observed at δ 3.9 which could be assigned to $C_3\alpha$-*H*. A double doublet for one proton appeared at δ 2.8 (J = 17.05 and 5.3 Hz) depicting the presence of $C_5\alpha$-*H*. The prominent peaks for angular and side-chain methyl protons were observed at δ 1.18, 0.97, 0.83 and 0.70. The ^{13}C NMR spectrum of compound **58** displayed

characteristic signal at δ 162 showing the presence of C=N while as the signals at δ 134, 120 and 57.7 were assigned to C_6, C_7 and C_3, respectively. Remaining carbon atoms were seen in accordance to the cholestane series. The structure of compound **58** was further supported by its mass spectrum in which the distinct molecular ion peak ($M^{+\cdot}$ 489/491) was found.

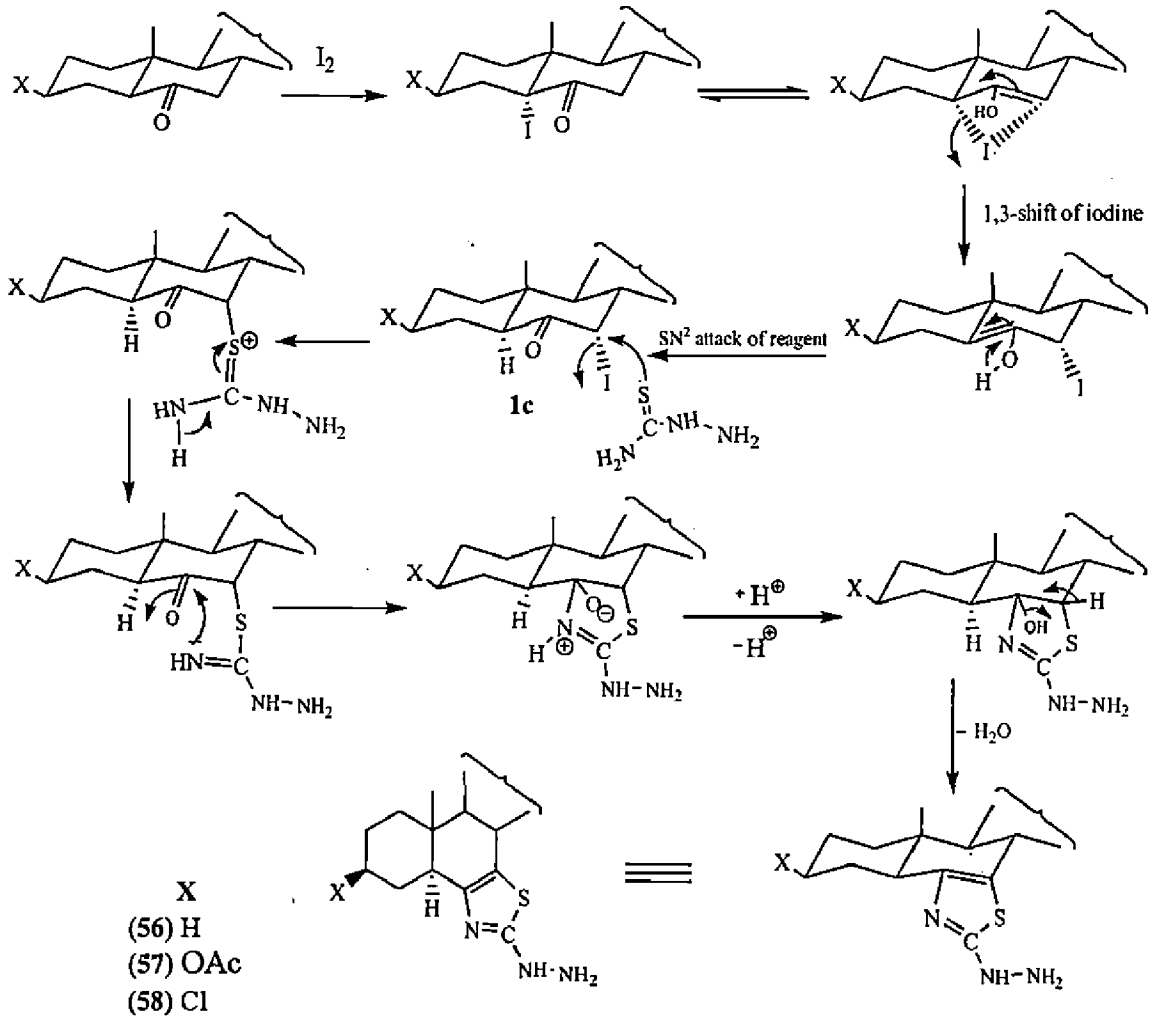
The mechanism for the formation of steroidal aminothiazole derivatives (**56-58**) can be explained by considering that during the reaction the α-iodoketone **1a** formed *in situ* undergoes allylic displacement of iodine *via* enolization and the subsequent attack of sulfur of thiosemicarbazide followed by cyclization leads to the formation of products **56-58** (**Scheme 4.2a**). An enol tautomeric form **1b** might be the driving force to accelerate the reaction towards product formation.



X
(**56**) H
(**57**) OAc
(**58**) Cl

Scheme 4.2a. Mechanism for the formation of steroidal aminothiazole derivatives (**56-58**) via the allylic displacement of iodine.

The formation of products **56-58** may also be explained by an alternate route considering that during the reaction 1, 3-shift of iodine from C_5 to C_7 leads to the formation of intermediate **1c** *in situ* followed by S_N^2 attack of sulfur of the reagent and subsequent cyclization provides the desired products **56-58** (**Scheme 4.2b**).



Scheme 4.2b. Mechanism for the formation of steroidal aminothiazole derivatives (**56-58**) via 1, 3-shift of iodine.

Work accepted;

Synthesis, characterization and in vitro anticancer activity of newly synthesized steroidal 6, 7-fused thiazoles, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Chemistry* (accepted)

Experimental

All the melting points were determined in degrees Celsius on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Perkin Elmer RXI Spectrophotometer and values are given in cm^{-1}. ^1H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse (400 MHz) instrument with TMS as internal standard and values are given in ppm (δ). Mass spectra were recorded on a JEOL SX 102/DA-6000 Mass Spectrometer. Thin layer chromatography (TLC) plates were coated with silica gel G and exposed to iodine vapors to check the homogeneity as well as the progress of reaction. Petroleum ether refers to a fraction of boiling point 60-80 °C. Sodium sulfate (anhydrous) was used as a drying agent.

The synthesis of 3β-chlorocholest-5-ene, cholest-5-ene, 6-nitrocholest-5-ene and 5α-cholestan-6-one is shown in **chapter 1**, page 23, 24. The synthesis of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and 3β-acetoxy-5α-cholestan-6-one is shown in **chapter 1**, page 24, 25. The synthesis of 3β-chloro-6-nitrocholest-5-ene and 3β-chloro-5α-cholestan-6-one is shown in **chapter 1**, page 25, 26.

Reaction of 5α-cholestan-6-one derivatives (50-52) with iodine and 2-aminothiophenol:

To a solution of 5α-cholestan-6-one derivatives (**50-52**) (1 mmol) in absolute ethanol (10 mL) was added 2-aminothiophenol/ thiosemicarbazide (1 mmol) and iodine (2 mmol) in the same solvent (25 mL) and the reaction mixture was refluxed for 13-21 h. The progress of the reaction was monitored by TLC. After completion of reaction the excess solvent was reduced to three fourths of the original volume under reduced pressure. Then it was cooled to room temperature, diluted with $Na_2S_2O_7$ solution and subsequently with water. The mixture was extracted with diethyl ether, washed with water and finally dried over anhydrous sodium sulfate. Evaporation of solvents and crystallization of the oily residue from methanol afforded corresponding products (**53-58**).

5α-Cholestano [5, 6 - b] benzothiazine (53):

Yield 83%; m.p. 152 °C; Analysis found: C 80.59, H 10.04, N 2.85%. $C_{33}H_{48}NS$ requires: C 80.12, H 9.86, N 2.63%; IR (KBr): ν_{max} 3062, 1600 (aromatic), 1628 (C=N), 1385 (C-N), 711 (C-S); ^1H NMR ($CDCl_3$): δ 6.43-6.24 (*m*, 4H, aromatic), 2.05 (*d*, 2H, C_4-H_2, J = 8.0 Hz), 2.04 (*d*, 2H, C_7-H_2, J = 4.4 Hz), 1.17 (*s*, 2H, C_{10}-CH_3), 0.75 (*s*, 3H, C_{13}-CH_3), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 163 (*C*=N), 149, 125, 124, 122.8, 122, 120 (aromatic carbons), 48 (C_3), 46 (C_{14}), 42.2 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}), 20 (C_{15}); MS: *m/z* 491 [$M^{+\cdot}$].

3β-Acetoxy-5α-cholestano [5, 6 - b] benzothiazine (54):

Yield 80%; m.p. 163 °C; Analysis found: C 76.45, H 9.35, N 2.55%. $C_{35}H_{51}NO_2S$ requires: C 76.17, H 9.08, N 2.32%; IR (KBr): ν_{max} 3060, 1603 (aromatic), 1714 ($OCOCH_3$), 1650 (C=N), 1388 (C-N), 1206 (C-O), 750 (C-S); ^{1}H NMR ($CDCl_3$): δ 6.33-6.28 (*m*, 4H, aromatic), 4.7 (*m*, 1H, $C_3\alpha$-*H*, *W½* = 15 Hz), 2.03 (*s*, 3H, $OCOCH_3$), 1.9 (*d*, 2H, C_7-H_2, *J* = 5.2 Hz), 1.8 (*d*, 2H, C_4-H_2, *J* = 8.0 Hz), 1.18 (*s*, 3H, C_{10}-CH_3), 0.70 (*s*, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 174 ($OCOCH_3$), 163 (*C*=N), 148, 129, 128, 126, 124, 122 (aromatic carbons), 73 (C_3), 46 (C_{14}), 44 (C_{13}), 42 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}); MS: *m/z* 549 [$M^{+\cdot}$].

3β-Chloro-5α-cholestano [5, 6 - b] benzothiazine (55):

Yield 85%; m.p. 146 °C; Analysis found: C 75.32, H 9.19, N 2.66%. $C_{33}H_{48}ClNS$ requires: C 75.07, H 9.03, N 2.61%; IR (KBr): ν_{max} 3058, 1598 (aromatic), 1626 (C=N), 1380 (C-N), 740 (C-Cl), 710 (C-S); ^{1}H NMR ($CDCl_3$): δ 6.43-6.24 (*m*, 4H, aromatic), 3.5 (*m*, 1H, $C_3\alpha$-*H*, *W* ½ = 17 Hz), 2.07 (*d*, 2H, C_7-H_2, *J* = 8.0 Hz), 1.87 (*d*, 2H, C_4-H_2, *J* = 4.8 Hz), 1.18 (*s*, 2H, C_{10}-CH_3), 0.75 (*s*, 3H, C_{13}-CH_3), 0.97 and 0.80 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 164 (*C*=N), 146, 127, 126, 125, 123, 122 (aromatic carbons), 59 (C_3), 47 (C_{14}), 46 (C_{13}), 42.6 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}), 20 (C_{15}), 17 (C_{16}); MS: *m/z* 525/527 [$M^{+\cdot}$].

2'-Hydrazinocholest-6-eno [4, 5 - d] thiazole (56):

Yield 73%; m.p. 129 °C; Analysis found: C 73.52, H 10.28, N 9.19%. $C_{28}H_{47}N_3S$ requires: C 73.47, H 10.19, N 9.13%; IR (KBr): ν_{max} 3376, 3328 (NH, NH_2), 1617 (C=C), 1557 (C=N), 1328 (C-N), 634 (C-S); ^{1}H NMR ($CDCl_3$): δ 6.2 (br s, 2H, NH_2, exchangeable with D_2O), 3.8 (s, 1H, N*H*, exchangeable with D_2O), 2.74 (dd, 1H, $C_5\alpha$-*H*, *J* =16.9 Hz, 5.5 Hz), 1.18 (s, 3H, C_{10}-CH_3), 0.70 (s, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 163 (*C*=N), 130 (C_6), 120 (C_7), 50 (C_3), 46 (C_{14}), 42.2 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}), 20 (C_{15}), 17 (C_{16}); MS: *m/z* 457 [$M^{+\cdot}$].

3β-Acetoxy-2'-hydrazinocholest-6-eno [4, 5 - d] thiazole (57):

Yield 82%; m.p. 136 °C; Analysis found: C 69.90, H 9.51, N 8.15 %. $C_{30}H_{49}N_3O_2S$ requires: C 69.84, H 9.39, N 8.11%; IR (KBr): ν_{max} 3395, 3310 (NH, NH_2), 1730 ($OCOCH_3$), 1625 (C=C), 1555 (C=N), 1320 (C-N), 1210 (C-O), 645 (C-S); ^{1}H NMR ($CDCl_3$): δ 6.8 (br s, 2H, NH_2, exchangeable with D_2O), 4.7 (m, 1H, $C_3\alpha$-*H*, *W½* =15 Hz), 4.4 (s, 1H, N*H*, exchangeable with D_2O), 2.7 (dd, 1H, $C_5\alpha$-*H*, *J* =15 Hz, 5 Hz), 2.03 (s, 3H, OAc), 1.18 (s,

3H, C_{10}-CH_3), 0.70 (s, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 171.2 ($OCOCH_3$), 163 (C=N), 132 (C_6), 120 (C_7), 70.2 (C_3), 46 (C_{14}), 44 (C_{13}), 42 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}), 20 (C_{15}), 17 (C_{16}); MS: *m/z* 515 [M^+].

3β-Chloro-2'-hydrazinocholest-6-eno [4, 5 - d] thiazole (58):

Yield 76%; m.p. 143 °C; Analysis found: C 68.43, H 9.36, N 8.54%. $C_{28}H_{46}ClN_3S$ requires: C 68.37, H 9.29, N 8.49%; IR (KBr): ν_{max} 3370, 3320 (NH, NH_2), 1622 (C=C), 1560 (C=N), 1323 (C-N), 745 (C-Cl), 635 (C-S); ^1H NMR ($CDCl_3$): δ 6.63 (br s, 2H, NH_2, exchangeable with D_2O), 4.45 (s, 1H, N*H*, exchangeable with D_2O), 3.9 (m, 1H, $C_3\alpha$-*H*, *W½* = 17 Hz), 2.8 (dd, 1H, $C_5\alpha$-*H*, *J* =17.05 Hz, 5.3 Hz), 1.18 (s, 3H, C_{10}-CH_3), 0.70 (s, 3H, C_{13}-CH_3), 0.97 and 0.83 (other methyl protons); ^{13}C NMR ($CDCl_3$): δ 162 (C=N), 134 (C_6), 120 (C_7), 57.7 (C_3), 46 (C_{14}), 45 (C_{13}), 42.6 (C_4), 39 (C_{10}), 35 (C_5), 26 (C_{19}), 24 (C_{11}), 22 (C_{18}), 20 (C_{15}), 17 (C_{16}); MS: *m/z* 489/491 [M^+].

References

References

1. B. Loev, M. F. Kormendy, *J. Org. Chem.*, **30**, 3163 (1965)
2. G. Prota, E. Ponsiglione, R. Ruggiero, *Tetrehedron*, **30**, 2781 (1974)
3. L. D. Corona, G. Signorelli, A. Pinzetta, G. Coppi, *Eur. J. Med. Chem.*, **27**, 419 (1992)
4. A. M. Farag, A. S. Shawali, M. S. Algharib, K. M. Dawood, *Tetrahedron*, **50**, 5091 (1994)
5. A. Napolitano, C. Costantini, O. Crescenzi, G. Prota, *Tertrahedron Lett.*, **35**, 6365 (1994)
6. V. L. D. M. Guarda, M. Perrissin, F. Thomasson, E. A. Ximenes, S. L. Galdino, I. R. Pitta, C. Luu-Duc, J. Barbe, *Eur. J. Med. Chem.*, **38**, 769 (2003)
7. A. A. Esmaili, M. Ghereghloo, M. R. Islami, H. R. Bijanzadeh, *Tetrahedron*, **59**, 4785 (2003)
8. J. Matysiak, *Bioorg. Med. Chem.*, **14**, 2613 (2006)
9. P. B. Madrid, W. E. Polgar, L. Tolla, M. J. Tanga, *Bioorg. Med. Chem. Lett.*, **17**, 3014 (2007)
10. M. Bakavoli, M. Nikpour, M. Rahimizadeh, M. R. Saberi, H. Sadeghian, *Bioorg. Med. Chem. Lett.*, **15**, 2120 (2007)
11. V. K. Tandon, H. K. Maurya, N. N. Mishra, P. K. Shukla, *Eur J. Med. Chem.*, **44**, 3130 (2009)
12. U. R. Pratap, D. V. Jawale, B. S. Londhe, R. A. Mane, *J. Mol. Cat. B*, **68**, 94 (2011)
13. M. Alam, S. Khan, M. S. Khan, *J. Chilean Chem. Soc.*, **53**, 1714 (2008)
14. A. Khan, M. Alam, M. Mushfiq, *Chinese Chem. Lett.*, **19**, 1027 (2008)
15. M. Kumar, K. Sharma, R. M. Samarth, A. Kumar, *Eur J. Med. Chem.*, **45**, 4467 (2010)
16. S. I. Zav'yalov, N. E. Kravchenko, G. I. Ezhova, L. B. Kulikova, A. G. Zavozin, O. V. Dorofeeva, *J. Pharm. Chem.*, **41**, 45 (2007)
17. K. Visvanathan, N. E. Davidson, *Oncology* (Williston Park), **17**, 335 (2003)
18. X. Li, S. M., Singh, J. Cote, S. Laplante, R. Veilleux, *J. Med. Chem.*, **38**, 1456, (1995)
19. Q. He, D. Jiang, *Leuk. Res.*, **23**, 369 (1999)
20. K. A. Latham, A. Zamora, H. Drought, S. Subramanian, A. Matejuk, H. Offner, E. F. Rosloniec, *J. Immunol.*, **17**, 5820 (2003)
21. D. Hosking, C. E. Chilvers, C. Christiansen, P. Ravn, R. Wasnich, P. Ross, M. McClung, A. Balske, D. Thompson, M. Daley, A. J. Yates, *N. Engl. J. Med.*, **338**, 485 (1998)
22. V. Gautam, M. Sharma, M. Panwar, N. Gautam, A. Kumar, I. K. Sharma, D. C. Gautam, *Phosphorus Sulfur, and Silicon*, **184**, 3090 (2009)
23. Y. Watanabe, K. Osanai, T. Nishi, N. Miyawaki, D. Shii, T. Honda, T. Shibano, *Bioorg. Med. Chem. Lett.*, **6**, 1923 (1996)
24. A. Niewiadomy, J. Matysiak, M. M. Karpinska, *Arch. Pharm. Chem. Life Sci.*, **11**, 224 (2011)

25. V. Cecchetti, V. Calderone, O. Tabarrini, S. Sabatini, E. Filipponi, L. Testai, R. Spogli, E. Martinotti, A. Fravolini, *J. Med. Chem.*, **46**, 3670 (2003)
26. T. Matsui, Y. Nakamura, H. Ishikawa, A. Matsuura, F. Kobayashi, *Jpn. J. Pharmacol.*, **64**, 115 (1994)
27. M. Kajino, K. Mizuno, H. Tawada, Y. Shibouta, K. Nishikawa, K. Meguro, *Chem. Pharm. Bull.*, **39**, 2888 (1991)
28. B. S. Rathore, M. Kumar, *Bioorg. Med. Chem.*, **14**, 5678 (2006)
29. K. D. Hargrave, F. K. Hess, J. T. Oliver, *J. Med. Chem.*, **26**, 1158 (1983)
30. S. Bondock, W. Khalifa, A. A. Fadda, *Eur. J. Med. Chem.*, **42**, 948 (2007)
31. P. K. Sharma, S. N. Sawhney, A. Gupta, G. B. Singh, *Indian J. Chem.*, **37B**, 376 (1998)
32. J. C. Jean, L. D. Wise, B. W. Caprathe, H. Tecle, S. Bergmeier, C. C. Humblet, T. G. Heffner, L. T. Meltzer, T. A. Pugsley, *J. Med. Chem.*, **33**, 311 (1990)
33. K. Tsuji, H. Ishikawa, *Bioorg. Med. Chem. Lett.*, **4**, 1601(1994)
34. F. W. Bell, A. S. Cantrell, M. Hoqberg, S. R. Jaskunas, N. G. Johansson, C. L. Jordan, M. D. Kinnick, P. Lind, J. M. Morin, R. Noreen, B. Oberg, J. A. Palkowitz, C. A. Parrish, P. Pranc, C. Sahlberg, R. J. Ternansky, R. T. Vasileff, L. Vrang, S. J. West, H. Zhang, X. X. Zhou, *J. Med. Chem.*, **38**, 4929 (1995)
35. N. Ergenc, G. Capan, N. S. Gunay, S. Ozkirimli, M. Gungor, S. Ozbey, E. Kendi, *Archiv der Pharmazie*, **332**, 343 (1999)
36. J. S. Carter, S. Kramer, J. J. Talley, T. Penning, P. Collins, M. J. Graneto, K. Seibert, C. M. Koboldt, J. Masferrer, B. Zweifel, *Bioorg. Med. Chem. Lett.*, **9**, 1171 (1999)
37. A. Badorc, M. F. Bordes, P. De Cointet, P. Savi, A. Bernat, A. Lalé, M. Petitou, J. P. Maffrand, J. M. Herbert, *J. Med. Chem.*, **40**, 3393 (1997)
38. J. Rudolph, H. Theis, R. Hanke, R. Endermann, L. Johannsen, F. U. Geschke, *J. Med. Chem.*, **44**, 619 (2001)
39. S. R. Pattan, N. S. Dighe, S. A. Nirmal, A. N. Merekar, R. B. Laware, H. V. Shinde, D. S. Musmade, *Asian J. Res. Chem.*, **2**, 196 (2009)
40. C. W. Shoppe, R. H. Jenkins, G. H. R. Summers, *J. Chem. Soc.*, 1657 (1958)
41. R. M. Dodson, B. Riegel, *J. Org. Chem.*, **13**, 424 (1948)
42. A. Windus, O. Dalmer, *Chem. Ber.*, **52**, 162 (1919)
43. C. N. R. Rao, R. Venkataragh, *Can. J. Chem.* **42**, 43 (1964)

Chapter-5

Biological evaluation and DNA binding studies

Theoretical

Steroids have been the important focus of research throughout the scientific history. But the recent past has seen an exhaustive focus of research being diverted towards these biologically important molecules. This is pertinently true of the rational semi-synthetic modifications of steroidal molecules. Probably, it is because of the various advantages associated with steroid based chemotherapeutics. These compounds turn out to be non-toxic, less vulnerable to multi-drug resistance (MDR) and highly bioavailable because of being capable of penetrating the cell wall.[1]

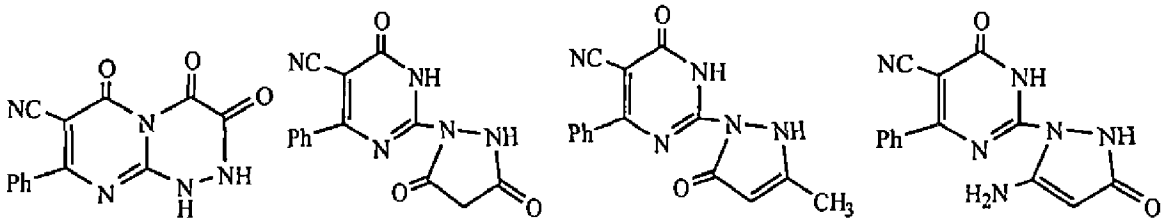
Steroid based antimicrobial agents continue to play a prominent role in those organisms which do not rely upon external supply of drugs to fight against pathogens[2] because the entire morbidity and mortality mostly in developing countries is due to these microbial infections[3] among which *Escherichia coli* is responsible for the most common and serious infectious diseases like invasive dysentery and diarrhoea.[4] The different microbes such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Salmonella typhimurium* have important effect on the human's mucosal health. The infection with these microorganisms may have a significant impact on huge demolition of host tissue and severe diseases.[5, 6]

Nitrogen containing steroids have the ability to regulate a variety of biological processes and thus are potential drug candidates for the treatment of a large number of diseases including breast cancer, prostate cancer, leukaemia, autoimmune diseases and osteoporosis.[7-11] So is the case with the nitrogen containing derivatives, pyrimidines, pyrans, pyrazoles, pyrazolones, benzothiazines and thiazoles which exhibit a broad spectrum of biological activities such as anticancer, antiviral, antibacterial, antifungal, antioxidant, anxiolytic and antidepressant. Furthermore, they possess anti-inflammatory and analgesic activities which are well documented in the literature.[12-20]

Antioxidants have gained much importance because of their potential as prophylactic and therapeutic agents in many diseases. Free radicals are constantly formed as a result of normal organ functions or excessive oxidative stress.[21] High levels of free radicals can cause damage to biomolecules such as lipids, proteins, enzymes and DNA in cells and tissues, resulting in mutations that can lead to malignancy. Damage to DNA by oxidative stress has been widely accepted as a major cause of cancer.[22] DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been observed in various tumours. The discovery of the role of free radicals in cancer, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders, aging and other diseases has led to new medical insight.[23, 24] Minimizing oxidative damage may be an important approach to

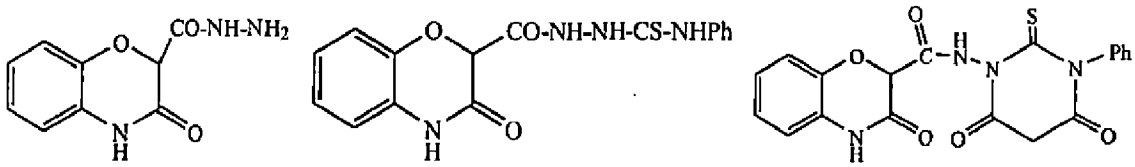
the primary prevention or treatment of these diseases, since antioxidants may stop the free-radical formation, or interrupt an oxidizing chain reaction. This had attracted a great deal of research interest in therapeutic antioxidant-based drug formulations. The development of heterocycles which are capable of scavenging free radicals, have been a great success.

El-zahar and co-workers[25] reported the synthesis of substituted pyrimidine derivatives (**1 a-d**). These compounds were tested for cytotoxicity against human liver carcinoma cell line (HepG2) during which all compounds were highly active as compared to 5-fluorouracil (5-Fu) with $IC_{50} < 10$ µg/mL. During the docking study the actual docking target sites was found and the negative bonding energy against amino acid residues of protein was calculated.



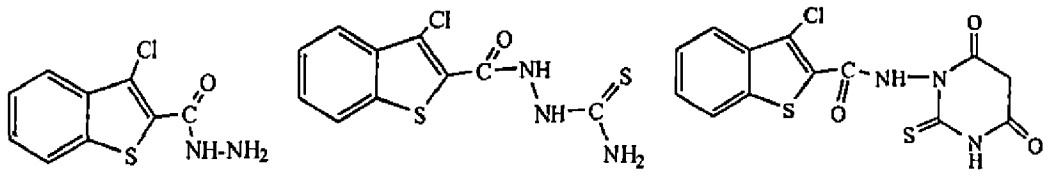
(1a) **(1b)** **(1c)** **(1d)**

Dabholkar and Gavande[26] reported the synthesis of substituted 1, 4-benzoxazine derivatives (**2 a-c**). The compounds were investigated for antibacterial activity against Gram positive as well as Gram negative bacteria with Ampicillin as standard drug during which the new compounds were found active with potential zone of inhibition.



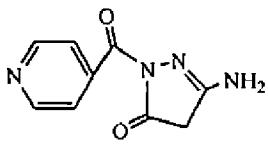
(2a) **(2b)** **(2c)**

Naganagowda and co-workers[27] also reported the synthesis of substituted thiophene derivatives (**3 a-c**). The compounds were screened for antimicrobial, analgesic and anti-helmintic activity during which the compounds were found to be biologically active.

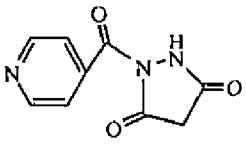


(3a) **(3b)** **(3c)**

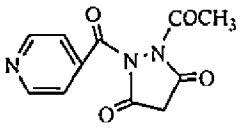
Parashar and co-workers[28] reported the synthesis and antimicrobial activity of pyrazole derivatives (**4 a-d**) during which all the compounds were found to be biologically active against bacteria as well as fungi especially against *B. subtitis*, *S. aureus* and *C. albicans*.



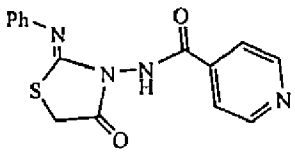
(4a)



(4b)

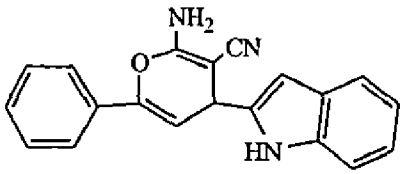


(4c)



(4d)

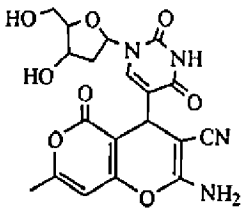
El-Latif and co-workers[29] reported the synthesis of 2-amino-4-(3-indolyl)-6-(3-pyridyl)-pyran-3-carbonitrile (**5**).



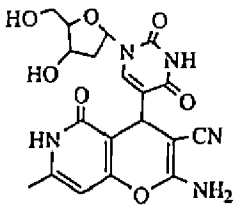
(5)

The compound and its derivatives were screened for antimicrobial, analgesic and anticonvulsant activity during which these were found to be biologically active in comparison with the biological standards.

Feng and co-workers[30] reported the synthesis of pyrano [3, 2 - *c*] pyridine nucleoside derivative (**6a**) and pyrano [4, 3 - *b*] pyran nucleoside derivative (**6b**) as potential antiviral and anti-leishmanial agents. Both the compounds were found biologically active by being effective at less concentration.

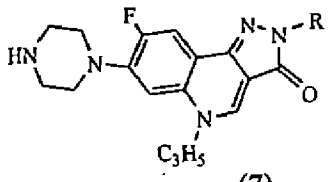


(6a)



(6b)

Devnath and Islam[31] reported the synthesis and anticancer studies of substituted quinolone derivatives (**7 a-c**). The IC_{50} obtained were 0.021, 0.019 and 0.023 μg/mL.



(7)

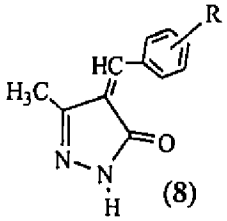
R

(a) Ph

(b) $C_6H_3(NO_2)_2$

(c) CS-NH_2

Mariappan and co-workers[32] reported the synthesis of substituted pyrazolones (**8 a-g**). The compounds were screened for analgesic and anti-inflammatory activity on albino mice, during which all the synthesized compounds were found to be biologically active.

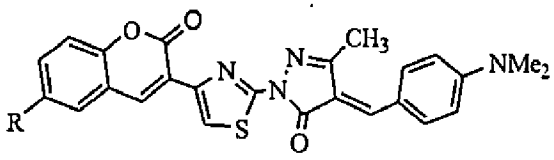


(8)

R

(a) $N(CH_3)_2$ (e) CH=CH-Ph
(b) $O(CH_3)_2$ (f) OMe
(c) Cl (g) OH
(d) NO_2

Chunduru and Rao[33] reported the one pot synthesis of 4-arylidene-3-methyl-1-[4-(2-oxo-2H-chromen-3-yl) thiazol-2-yl]-1H-pyrazol-5(4H)-one derivatives (**9 a-d**). The pyrazoles were screened for anticancer activity during which they showed $IC_{50} < 15$ µg/mL while as in antimicrobial activity these compounds depicted effective zone of inhibition.

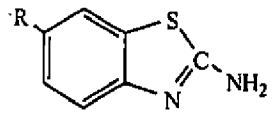


(9)

R

(a) H
(b) Br
(c) NO_2
(d) OCH_3

Kumar and co-workers[34] reported the synthesis of benzothiazole derivatives (**10 a-d**). The compounds were tested for antioxidant (LPO and GSH) and radical scavenging activities (DPPH and ABTS assays) during which compounds showed potential antioxidant behavior.

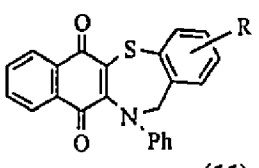


(10)

R

(a) CH_3 (c) OCH_3
(b) CF_3 (d) Br

Tandon and co-workers[35] reported the synthesis of dihydrobenzo [*f*] naphtha [2, 3 - *b*] [1, 4] thiazepindiones (**11 a-d**) as antimicrobial agents. These compounds showed activity against *Sporothrix schenckii* (MIC_{50}=1.56 µg/mL), significant profile against *Candida albicans* (MIC_{50}=1.56 µg/mL), *Cryptococcus neoformans* (MIC_{50}= 0.78 µg/mL) and *Trichophyton mentagraphytes* (MIC_{50}=1.56 µg/mL) and same antifungal activity when compared with Amphotericin-B against *C. neoformans* (MIC_{50}= 0.78 µg/mL).

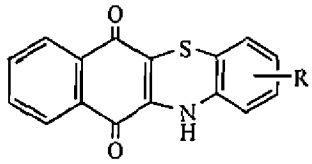


(11)

R

(a) H
(b) OMe
(c) NO_2
(d) C_4H_{12}

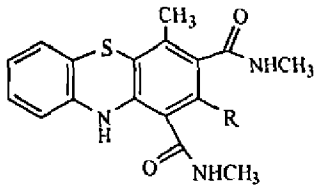
Tandon and co-workers[36] also reported the synthesis of 6H-benzo[*b*] phenothiazine-6, 11(12H)-dione derivatives (**12 a-c**) as potential antiproliferative and antifungal agents. Compounds **12b** and **12c** were found to possess most potent antiproliferative and cell killing ability against human cervical cancer (HeLa) cells. These compounds exhibited MIC_{50}=5.0 μg/mL against the strains of *C. albicans*, *T. mentagraphytes*, *S. schenckii* and *C. neoformans*.



R
(a) H
(b) OMe
(c) C_4H_{12}

(12)

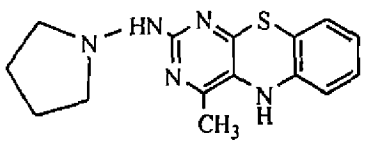
Sadanandam *et al.*[37] reported the synthesis of 2-aryl-N, N', 4-trimethyl-10H-pheno thiazine-1, 3-dicarboxamides (**13 a-c**) as enzyme inhibitors for inflammatory diseases. The results showed that these compounds exhibited promising target specific enzyme inhibition against phosphodiesterase, prostaglandin dehydrogenase and superoxide dismutase activity depending on steric factors of the molecules.



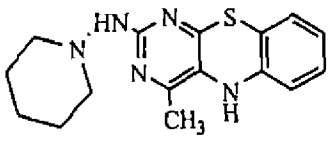
R
(a) C_6H_5
(b) $C_6H_2Cl_2$
(c) $C_7H_5O_2$

(13)

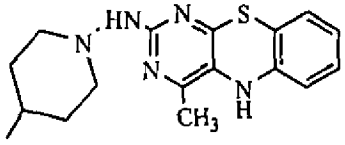
Bakavoli and co-workers[38] reported the synthesis of pyrimido [4, 5 - *b*] [1, 4] benzothiazines (**14 a-e**) as potent 15-lipoxygenase inhibitors. Compounds showed the best IC_{50} of 15-LO inhibition (IC_{50} = 18 and 34 μM). All compounds were docked into 15-LO. As a result, the sulfur atom was oriented toward the iron atom of the active site of 15-LO.



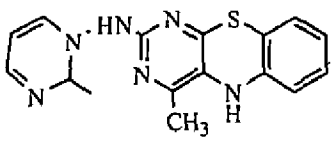
(14a)



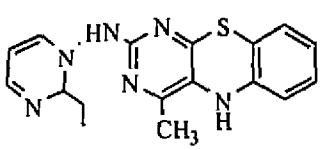
(14b)



(14c)



(14d)



(14e)

Antimicrobial activity

Experimental

Materials and equipments:

Tested microbes used in the study were *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, *Candida albicans*, *Aspergillus fumigatus*, *Trichophyton mentagrophytes* and *Penicillium marneffei*. The microbial strains were provided by American Type Culture Collection (ATCC). The images were taken from Axio Imager 2 fluorescent microscope. The standard drugs used in the study were Ciprofloxacin, Chloramphenicol, Griseofulvin and Nystatin which were being purchased from Merk (India).

***In vitro* antibacterial activity:**

The *in vitro* antibacterial activity of new steroidal derivatives was done by the disk diffusion method and zones were measured by Halo Zone Test.[39, 40] The MIC of synthesized compounds against bacterial and fungal strains was checked by micro dilution test and results were observed visually and spectrophotometrically. The new compounds were screened for antibacterial activity against the cultures of *Escherichia coli* (ATCC-25922), *Staphylococcus aureus* (MRSA +Ve) (ATCC-25923), *Pseudomonas aeruginosa* (ATCC-27853), *Streptococcus pyogenes* (ATCC 19615) and *Klebsiella pneumoniae* (Clinical isolate) Standard inoculums (1-2 × 10^7 c.f.u./mL 0.5 McFarland standards) was introduced on to the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculums. The discs measuring 6 mm in diameter were prepared from Whatman no. 1 filter paper and sterilized by dry heat at 140 °C for 1 h. The sterile discs previously soaked in a known concentration of the test compounds were placed in nutrient agar medium. Solvent and growth controls were kept. Ciprofloxacin and Chloramphenicol were used as positive controls while the disk poured in DMSO was used as negative control. The plates were inverted and incubated for 24 h at 37 °C. The susceptibility was assessed on the basis of diameter of zone of inhibition against Gram-positive and Gram-negative strains of bacteria. Inhibition zones were measured and compared with the controls.

Minimum inhibitory concentrations (MICs) were determined by broth dilution technique. The nutrient broth which contained logarithmic serially two fold diluted amount of test compound and controls were inoculated with approximately 5 × 10^5 c.f.u./mL of actively dividing bacteria cells. The cultures were incubated for 24 h at 37 °C and the growth was monitored visually and spectrophotometrically. The lowest concentration (highest dilution)

required to arrest the growth of bacteria was regarded as minimum inhibitory concentration (MIC). To obtain the minimum bacterial concentration (MBC), 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 18-24 h of incubation at 35 °C. MBC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed.

***In vitro* antifungal assay:**

The antifungal assay was studied against the cultures of *Candida albicans* (ATCC-10231), *Aspergillus fumigatus* (ATCC-1022), *Trichophyton mentagrophytes* (ATCC-9533) and *Penicillium marneffei* (recultured) in DMSO by agar diffusion method.[41, 42] Sabourands agar media was prepared by dissolving peptone (1 g), D-glucose (4 g) and agar (2 g) in distilled water (100 mL) and adjusting pH to 5.7. Normal saline was used to make a suspension of spore of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 mL saline to get a suspension of corresponding species. Agar media (20 mL) was poured into each Petri dish. Excess of suspension was decanted and the plates were dried by placing in an incubator at 37 °C for 1 h using an agar punch, wells were made and each well was labelled. A control was also prepared in triplicate and maintained at 37 °C for 3-4 days. The fungal activity of compounds was compared with Griseofulvin and Nystatin as standard drugs. Inhibition zones were measured and compared with the controls. The nutrient broth which contained logarithmic serially two fold diluted amount of test compound and controls was inoculated with approximately 1.6 - 6 × 10^4 c.f.u./mL.

The cultures were incubated for 48 h at 35 °C and the growth was monitored. The lowest concentration (highest dilution) required to arrest the growth of fungus was regarded as minimum inhibitory concentration (MIC). The nutrient broth, which contained logarithmic serially two fold diluted amount of test compound and controls were inoculated with approximately 5 × 10^5 c.f.u./mL of actively dividing fungal cells. The cultures were incubated for 24 h at 37 °C and the growth was monitored visually and spectrophotometrically. To obtain the minimum fungicidal concentration (MFC), 0.1 mL volume was taken from each tube and spread on agar plates. The number of c.f.u. was counted after 48 h of incubation at 35 °C. MFC was defined as the lowest drug concentration at which 99.9% of the inoculums were killed.

Results and discussion

[4', 6'-Dioxo-2'-thioxo-1H-pyrimidin-1-yl]6-imino-5α-cholestane derivatives (94-96)

***In vitro* antibacterial activity:**

The antibacterial screening data of steroidal pyrimidines (refer to **chapter 1** for synthesis) revealed that all the tested compounds (**94-96**) showed moderate to good antibacterial activity against *S. pyogenes*, *S. aureus* and *E-coli* species. In general, all the compounds (**94-96**) were more effective against Gram positive bacteria as compared to Gram negative bacteria. The potential zone of inhibition was shown by compound **94** i.e. 17.3 mm against *S. pyogenes* with respect to the standard drug, Ciprofloxacin i.e. 23.0 mm against the same strain. Compound **94** also showed potential behavior against *K. pneumoniae* by depicting zone of inhibition i.e. 16.1 mm with respect to the Ciprofloxacin i.e. 19.0 mm. The maximum zone of inhibition shown by compound **95** was 16.6 mm against *S. aureus* (*MRSA* +ve) with respect to the standard drug, Ciprofloxacin i.e. 22.0 mm against the same strain. The potential zone of inhibition shown by compound **96** was 16.5 mm against *S. pyogenes* with respect to the standard drug, Ciprofloxacin i.e. 23.0 mm against the same strain. The bacterial zones of inhibition of steroidal pyrimidines (**94-96**) against all strains are given in **Table 1.**

Compounds	Diameter of zone of inhibition (mm)				
	Gram positive bacteria		Gram negative bacteria		
	S. pyogenes	*MRSA**	*P. aeruginosa*	*K. pneumoniae*	*E. coli*
94	17.3±0.2	16.6±0.4	19.4±0.6	16.1±0.4	16.7±0.4
95	16.2±0.3	16.6±0.2	18.3±0.4	15.2±0.3	16.6±0.4
96	16.5±0.4	14.9±0.5	17.8±0.2	13.7±0.2	14.7±0.2
Ciprofloxacin	23.0±0.2	22.0±0.2	32.0±0.3	19.0±0.2	27.0±0.2
DMSO	-	-	-	-	-

Table 1. Showing the zone of inhibition of compound **94-96** with given bacterial strains

The MICs of almost all the compounds were found to be two fold or four fold higher than MIC's of the standard drug, except compound **94** whose MIC was found to be same as that of standard drug i.e. 12.5 with respect to the *S. pyogenes*. The MIC's and MBC's are given in **Table 2.** From the **Table 2** it is also clear that the lowest concentration at which compound **94** inhibited the visible growth of bacteria is 12.5 and 25 µg/mL while as compound **95** and **96** inhibited the growth of bacteria at lowest concentration of 25 and 50 µg/mL against the given bacterial strains.

Compounds	Gram positive bacteria				Gram negative bacteria					
	S. pyogenes		*MRSA**		*P. aeruginosa*		*K. pneumoniae*		*E-coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
94	12.5	25	25	50	25	50	25	50	25	50
95	25	50	25	50	25	50	25	100	50	100
96	25	100	25	100	50	100	50	100	50	100
Ciproflxn.	12.5	12.5	6.25	12.5	12.5	25	6.25	25	6.25	12.5

Table 2. Showing the MIC's and MBC's of compound **94-96** with given bacterial strains

***In vitro* antifungal activity:**

The steroidal pyrimidines (**94-96**) (refer to **chapter 1** for synthesis) showed potential antifungal activity and in particular compound **94** was found active almost equivalent to the Griseofulvin against *C. albicans*[1], *A. fumigatus*[2], *T. mentagrophytes*[3] and *P. marneffei*[4] by showing maximum zones of inhibition. The MFC of few compounds was found to be the same as MFC of standard drug but in most of the compounds it was two or three or four folds higher than the corresponding MFC results of Griseofulvin. Compound **94** exhibited potential antifungal activity among the three steroid derivatives as its activity is nearly equivalent to Griseofulvin against *C. albicans* strain with zone of inhibition 27.8 ± 0.3 mm and MIC of 12.5 μg/mL. Compound **95** also exhibited potential antifungal activity against *C. albicans* by showing diameter of zone of inhibition 25.7 mm with respect to the Griseofulvin 30.0 mm. The fungal zones of inhibition of the tested compounds are given in **Table 3**. From the **Table 4** it is clear that the MIC's and MFC's of all the compounds were found to be two to four folds higher than that of the Griseofulvin.

	Diameter of zone of inhibition (mm)			
Compounds	CA[1]	AF[2]	TM[3]	PM[4]
94	27.8±0.3	22.7±0.4	19.6±0.2	17.5±0.2
95	25.7±0.4	21.4±0.3	18.2±0.5	15.1±0.4
96	23.5±0.3	19.8±0.4	17.2±0.6	12.9±0.4
Griseofulvin	30.0±0.2	27.0±0.2	24.0±0.3	20.0±0.5
DMSO	-	-	-	-

Table 3. Showing the zone of inhibition of compound **94-96** with different fungal strains

Comp.	CA[1]		AF[2]		TM[3]		PM[4]	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
94	12.5	25	12.5	25	12.5	25	12.5	50
95	12.5	50	12.5	50	25	50	25	50
96	25	50	25	50	25	100	25	100
Griseofulvin	6.25	25	12.5	12.5	6.25	25	12.5	25

Table 4. Showing the MIC's and MFC's of compound **94-96** with different fungal strains

2'-Amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran derivatives (63-65)

***In vitro* antimicrobial activity:**

The steroidal pyrans (**63-65**) (refer to **chapter 2** for synthesis) were screened for antibacterial activity against *E-coli* (ATCC-25922), *S. aureus* (MRSA +Ve) (ATCC-25923), *P. aeruginosa* (ATCC-27853), *S. pyogenes* (ATCC 19615) and *K. pneumoniae* (Clinical isolate). Activity of these compounds was studied by the disk diffusion method and zones were measured by Halo Zone Test.[39, 40] The antibacterial screening data (**Table 5** and **Table 6**) revealed that the compound **63-65** showed good antibacterial activity against *S. pyogenes*, *S. aureus* and *E. coli* species. Compound **63** afforded good zone of inhibition i.e. 21.2 mm against *S. pyogenes* with respect to the standard drug, Ciprofloxacin i.e. 24.0 nm. Except MIC of compound **63** against *S. pyogenes*, all the compounds showed MIC and MBC two to four fold higher than that of Ciprofloxacin.

Comp.	Diameter of zone of inhibition (mm)				
	Gram positive bacteria		Gram negative bacteria		
	S. pyogenes	*MRSA**	*P. aeruginosa*	*K. pneumoniae*	*E-coli*
63	21.2±0.4	18.7±0.4	26.1±0.3	17.2±0.3	22.1±0.2
64	19.4±0.2	17.3±0.3	23.1±0.2	15.1±0.4	20.1±0.4
65	19.2±0.4	17.2±0.6	22.1±0.3	15.1±0.3	19.1±0.3
Ciprofloxacin	24.0±0.3	25.0±0.4	32.0±0.3	18.0±0.2	26.0±0.4
DMSO	-	-	-	-	-

Table 5. Showing the zone of inhibition of compound **63-65** with different bacterial strains

Compounds	Gram positive bacteria				Gram negative bacteria					
	S. pyogenes		*MRSA**		*P. aeruginosa*		*K. pneumoniae*		*E. coli*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
63	12.5	25	25	50	25	50	25	50	25	50
64	25	50	100	100	50	100	50	100	50	100
65	25	50	50	100	50	100	50	100	50	100
Ciprofloxacin	12.5	50	12.5	50	12.5	25	25	50	12.5	50

Table 6. Showing the MIC's and MFC's of compound **63-65** with given bacterial strains

***In vitro* antifungal activity:**

Antifungal activity of steroidal pyrans (**63-65**) (refer to **chapter 2** for synthesis) was done against the cultures of *C. albicans*[1] (ATCC-10231), *A. fumigatus*[2] (ATCC-1022), *T. mentagrophytes*[3] (ATCC -9533) and *P. marneffei*[4] (recultured) in DMSO by agar diffusion method.[41, 42] The antifungal screening data (**Table 7** and **Table 8**) revealed that all the compounds showed good antifungal activity against *C. albicans*, *A. fumigatus* and *P. marneffei* fungal strains and depicted potential zones of inhibition with respect to positive control. All the compounds showed MIC and MFC two to four fold higher than MIC and MFC of the standards. Compound **63** showed maximum zones of inhibition i.e. 21.2 17.7, 16.3, 14.9 mm against *C. albicans*, *A. fumigatus*, *T. mentagrophytes* and *P. marneffei*, respectively with respect to Greseofulvin among all the steroidal pyrans.

	Diameter of zone of inhibition (mm)			
Compounds	CA[1]	AF[2]	TM[3]	PM[4]
63	20.6±0.2	17.7±0.3	16.3±0.2	14.9±0.4
64	18.8±0.4	15.6±0.2	14.3±0.6	12.9±0.2
65	18.7±0.5	15.4±0.3	13.5±0.6	12.7±0.6
Greseofulvin	30.0±0.2	27.0±0.2	24.0±0.3	20.0±0.5
DMSO	-	-	-	-

Table 7. Showing the zone of inhibition of compound **63-65** with given bacterial strains

Comp.	CA[1]		AF[2]		TM[3]		PM[4]	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
63	12.5	25	25	50	12.5	25	25	50
64	25	50	25	50	25	50	25	100
65	25	100	50	100	100	100	50	100
Standard	6.25	12.5	12.5	25	6.25	25	12.5	25

Table 8. Showing the MIC's and MFC's of compound **63-65** with given fungal strains

Cholest-6[5'-amino-1', 2'-dihydro pyrazol-3-one-1'-yl] 5-ene derivatives (73-75)

***In vitro* antibacterial activity:**

The steroidal pyrazolones (**73-75**) (refer to **chapter 3** for synthesis) were screened against the cultures of *S. pyogenes* (ATCC-29213), *S. aureus* (ATCC-25923), *P. aeruginosa* (ATCC-27853) and *E-coli* (ATCC-25922) by disc diffusion method and zones were measured by Halo Zone Test.[39, 40] The antimicrobial screening data (**Table 9** and **Table 10**) suggests that steroidal pyrazolones (**73-75**) exhibited varying degree of inhibitory effects on the growth of bacterial and fungal strains. In antibacterial activity, compound **73** and **74** showed larger zone of inhibition (18.2 and 19.5 mm) in comparison with Chloramphenicol (21.5 mm) against *S. pyogenes* while as they showed zone of inhibition 17.3 and 16.1 mm against *S. aureus* and *P. aeruginosa*, respectively in comparison with Chloramphenicol 20.6 and 22.2 against the given strains. The confocal microscopic diagrams of zones of inhibition are shown in **Fig. 1**.

	Zone of Inhibition (mm)			
Compounds	*S. aureus*	*S. pyogenes*	*P. aeruginosa*	*E-coli*
73	17.3 ± 0.6	18.2 ± 0.2	15.4 ± 0.4	13.9 ± 0.6
74	17.3 ± 0.3	19.5 ± 0.1	16.1 ± 0.3	14.9 ± 0.2
75	15.8 ± 0.4	13.7 ± 0.5	14.6 ± 0.5	15.4 ± 0.5
Chloramphenicol	20.6 ± 0.5	21.5 ± 0.4	22.2 ± 0.8	19.0 ± 0.2
DMSO	-	-	-	-

Table 9. The zones of inhibition of shown by the compound **73-75** in antibacterial activity

Work published as;
Structural, optical and antimicrobial studies of 3*β*-acetoxycholest-5-ene, 3*β*-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones. Shamsuzzaman, **Ayaz Mahmood Dar**, et al., ***J. Taibah University for Science***, (Elsevier) *http*://dx.doi.org/10.1016/j.jtusci.2013.08.003 (in press)

***In vitro* antifungal activity:**

For antifungal screening, *C. albicans* (ATCC-10231), *A. fumigates* (ATCC-1022), *T. mentagrophytes* (ATCC-9533) and *P. marneffei* (ATCC-18224) were recultured in DMSO by agar diffusion method.[41, 42] The pyrazolones **73-75** (refer to **chapter 3** for synthesis) also exhibited varying degree of inhibitory effects on the growth of fungal strains. The compounds showed moderate to potential antifungal activity and in particular compound **74** showed large zone of inhibition (26.2 mm) in comparison to Nystatin (29.0 mm) against *C. albicans*. Compound **73** also showed large zone of inhibition (23.2 mm) in comparison to Nystatin (29.0 mm) against *T. mentagrophytes*.

	Zone of Inhibition (mm)			
Compounds	*C. albicans*	*T. mentagrophytes*	*P. marneffei*	*A. fumigatus*
73	24.2 ± 0.2	23.2 ± 0.6	16.2 ± 0.3	15.1 ± 0.2
74	26.2 ± 0.5	22.1 ± 0.5	14.2 ± 0.2	13.3 ± 0.4
75	23.6 ± 0.6	21.6 ± 0.4	13.9 ± 0.5	16.2 ± 0.2
Nystatin	29.0 ± 0.5	29.0 ± 0.5	19.5 ± 0.5	19.5 ± 0.5
DMSO	-	-	-	-

Table 10. The zones of inhibition of compound **73-75** in antifungal activity

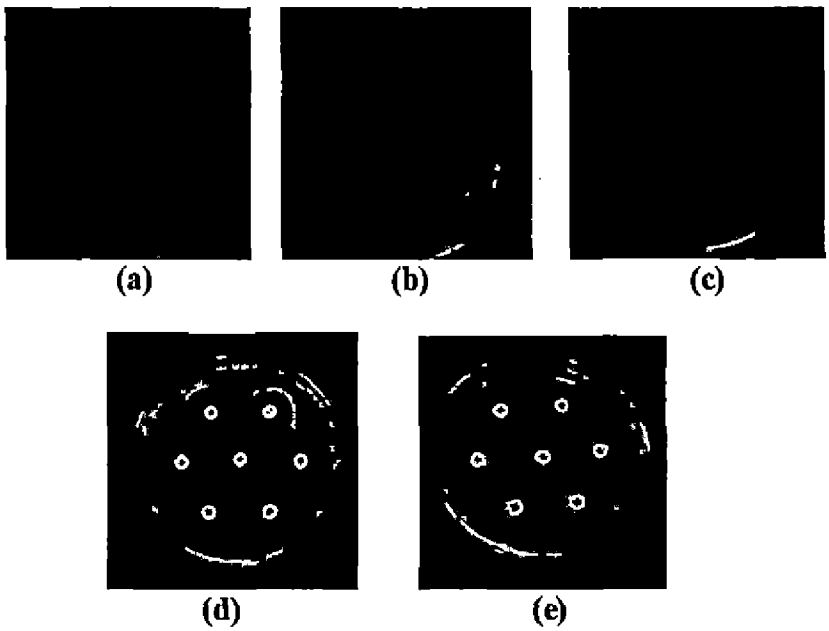


Fig. 1. Diagram showing zones created by the steroidal pyrazolone (**74**) against the strains: *P. Aeruginosa* (**a**), *S. aureus* (**b**), *A. fumigatus* (**c**), *S. pyogenes* (**d**) *C. albicans* (**e**).

5*α*-Cholestano [5, 6 - *b*] benzothiazine derivatives (53-55)

***In vitro* antimicrobial activity:**

Antimicrobial activity of steroidal benzothiazines (**53-55**) (refer to **chapter 4** for synthesis) was studied by the disk and agar diffusion method.[39-42] The screening data obtained after treating different microbial strains with test doses of the starting ketones (**50-52**) and steroidal benzothiazines (**53-55**) are given in **Table 11-14**. It is clear that the compound **53-55** showed moderate to good increase in antimicrobial activity than the starting compounds (**50-52**). In antibacterial activity, compound **54** and **53** showed potential inhibition zones against *E-coli* strain. Even the compound **54** was found to be more potent than Chloramphenicol, in case of *E-coli* strain. As shown in **Table 11**, compound **54** showed potential zones of inhibition *i.e.* 18.6, 19.6 and 21.5 mm against *S. aureus*, *S. pyogenes* and *P. aeruginosa*, respectively in comparison with the Chloramphenicol. The compound **53** also showed zone of inhibition *i.e.* 19.4 mm against the *E-coli* strain which is almost equal to that of Chloramphenicol *i.e.* 20.0 mm against the same strain. The MIC's of starting ketones (**50-52**) and benzothiazines (**53-55**) with different bacterial strains are given in **Table 12** and it is clear from the data that MIC's of steroidal ketones (**50-52**) are two to four fold higher than those of benothiazine derivatives (**53-55**) suggesting that compound **53-55** are active at lower concentration than those of compounds **50-52**. From the **Table 12** it is clear that the lowest concentration at which compound **54** inhibited the visible growth of bacteria is 32 and 64 µg/mL while as compound **55** and **53** inhibited the growth of bacteria at 32, 64 and 128 µg/mL.

	Zone of Inhibition (mm)			
Compounds	*S. aureus*	*S. pyogenes*	*P. aeruginosa*	*E-coli*
50	12.5 ± 0.4	11.6 ± 0.5	10.5 ± 0.5	10.2 ± 0.5
51	14.3 ± 0.4	12.2 ± 0.4	12.6 ± 0.2	14.2 ± 0.4
52	12.2 ± 0.2	10.3 ± 0.2	10.4 ± 0.4	11.2 ± 0.2
53	18.6 ± 0.1	15.1 ± 0.6	18.8 ± 0.4	19.4 ± 0.6
54	18.6 ± 0.2	19.6 ± 0.4	21.5 ± 0.5	23.2 ± 0.2
55	19.5 ± 0.4	15.3 ± 0.8	18.2 ± 0.4	15.6 ± 0.6
Chloramphenicol	21.6 ± 0.5	22.5 ± 0.4	25.2 ± 0.8	20.0 ± 0.2
DMSO	-	-	-	-

Table 11. Showing the zone of inhibition of starting steroidal ketones **50-52** and steroidal benzothiazines **53-55** with given bacterial strains

	MIC (μg/mL)						
Strains	**50**	**51**	**52**	**53**	**54**	**55**	Chloramphenicol
S. aureus	256	64	128	32	32	32	32
S. pyogenes	256	64	128	64	32	64	32
P. aeruginosa	128	128	128	128	64	64	32
E-coli	128	64	256	32	32	128	32

Table 12. Showing the MIC's of starting steroidal ketones (**50-52**) and steroidal benzothiazines (**53-55**) with given bacterial strains

It is clear also from the antifungal screening data that the steroidal benzothiazines (**53-55**) showed moderate to good increase in antifungal activity than the starting steroidal ketones (**50-52**). In antifungal activity, compound **54** and **55** showed potential inhibition zones against *P. marneffei* strain. But the inhibition zone of compound **55** is larger than compound **54** against *P. marneffei* in comparison with standard drug, Nystatin. As shown in **Table 13**, compound **54** showed potential zones of inhibition *i.e.* 24.5, 21.4, 16.6 and 15.1 mm against *C. albicans*, *T. mentagrophytes*, *P. marneffei* and *A. fumigatus*, respectively in comparison with the Nystatin. The compound **55** and **53** also showed influential zone of inhibition *i.e.* 17.4 and 24.2 mm against the *P. marneffei* and *T. mentagrophytes* strains which are very close to that of Nystatin *i.e.* 19.5 and 29.0 mm, respectively. The MIC's of starting steroidal ketones (**50-52**) and benzothiazines (**53-55**) with different fungal strains are given in **Table 14** and it is clear from the data that MIC's of steroidal ketones are two to four fold higher than those of benothiazine derivatives (**53-55**) revealing that compound **53-55** are active against different fungal strains at lower concentration than those of compound **50-52**. From the **Table 14** it is also clear that the lowest concentration at which compound **54** and **55** inhibited the visible growth of fungi is 32 μg/mL and 64 μg/mL while as compound **53** inhibited the growth of fungi at lower concentration *i.e.* 32 μg/mL against the different fungal strains.

Work published;

Anticancer and antimicrobial evaluation of newly synthesized steroidal 5, 6 fused benzothiazines. Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Arabian Journal of Chemistry*, http://dx.doi.org/10.1016/j.arabjc.2013.06.027 (in press)

Zone of Inhibition (mm)				
Compounds	*C. albicans*	*T. mentagrophytes*	*P. marneffei*	*A. fumigatus*
50	14.2 ± 0.5	11.2 ± 0.4	12.1 ± 0.2	10.1 ± 0.3
51	13.1 ± 0.5	14.1 ± 0.5	11.3 ± 0.5	11.5 ± 0.2
52	12.2 ± 0.2	09.2 ± 0.2	10.1 ± 0.5	09.2 ± 0.4
53	23.5 ± 0.2	24.2 ± 0.5	15.8 ± 0.2	16.0 ± 0.5
54	24.5 ± 0.3	21.4 ± 0.6	16.6 ± 0.3	15.1 ± 0.4
55	19.1 ± 0.4	18.2 ± 0.4	17.4 ± 0.5	14.4 ± 0.2
Nystatin	29.0 ± 0.5	29.0 ± 0.5	19.5 ± 0.5	19.5 ± 0.5
DMSO	-	-	-	-

Table 13. Showing the zone of inhibition of starting steroidal ketones (**50-52**) and steroidal benzothiazines (**53-55**) with different fungal strains

MIC (µg/mL)							
Strains	**50**	**51**	**52**	**53**	**54**	**55**	Nystatin
C. albicans	128	256	256	32	32	64	32
T. mentagrophytes	256	128	256	32	64	64	32
P. marneffei	128	256	128	32	32	32	32
A. fumigatus	256	128	256	32	32	32	32

Table 14. Showing the MIC's of starting steroidal ketones (**50-52**) and steroidal benzothiazines (**53-55**) with different fungal strains

Anticancer activity

Experimental

Materials and equipments:

The human cancer cell lines used for the cytotoxicity experiment were SW480, A549, HepG2, HL-60, MCF7, HeLa, A545, HT-29 and DU145 which were obtained from National Cancer Institute (NCI), biological testing branch, Federick Research and Development Centre, USA. The treated and control cancer cells were viewed with a FluoView FV1000 (Olympus, Tokyo, Japan) confocal laser scanning microscope (CLSM) equipped with argon and HeNe lasers. 2-Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Merck (India). Single cell electrophoresis (comet assay) was studied with fluorescent microscope (Olympus BX-51, Japan). Supercoiled plasmid pBR322 DNA was purchased from GeNei (India) and was used for the agarose gel experiment without further purification.

Anticancer activity:

Cell lines and culture conditions: Human cancer cell lines used for the study were A545 (lung carcinoma cells)/ATCC (CRL-2579), MCF7 (breast cancer cells)/ATCC (HTB-22), HeLa (cervical cancer cells)/ATCC (CCL-2), HL-60 (Leukaemia cells)/ATCC (CCL-240), SW480 (colon adenocarcinoma cells)/ATCC (CCL-228), HepG2 (hepatic carcinoma cells)/ATCC (CRL-8065), HT-29 (colon cancer cells)/ATCC (HTB-38), DU145 (pancreatic cancer cells)/ ATCC (HTB-81) and A549 (lung carcinoma cells)/ATCC (CCL-185) were obtained from National Cancer Institute (NCI), biological testing branch, Federick Research and Development Centre, USA. SW480, A549, A545, HL-60, HT29, DU145 and HepG2 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 10U penicillin and 100 µg/mL streptomycin at 37 °C with 5% CO_2 in a humidified atmosphere. HeLa and MCF7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplanted with FCS and antibiotics as described above for RPMI 1640. The non-cancer cells i.e. 184B5 and MCF10A breast cells were maintained in mammary epithelial basal medium supplemented with an MEGM mammary epithelial singlequot kit (Cambrex). NL-20 (normal lung cells), HPC (normal pulp cells), HPLF (periodontal ligament fibroblasts) were grown at 37 °C with 5% CO_2, 95% air under the humidified conditions. Fresh medium was given every second day and on the day before the experiments were done. Cells were passaged at preconfluent densities, using a solution containing 0.05% trypsin and 0.5 mM EDTA.

Cell viability assay (MTT): The *in vitro* cytotoxicity was measured using the MTT assay. The assay was carried out according to known protocol.[43, 44] Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well. After 24 h

incubation at 37 °C under a humidified 5% CO_2 to allow cell attachment, the cells in the wells were respectively treated with target compounds at various concentrations for 48 h. The concentration of DMSO was always kept below 1.25%, which was found to be non-toxic to the cells. A solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), was prepared at 5 mg/mL in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4) and 20 µL of this solution was added to each well. After incubation for 4 h at 37 °C in a humidified incubator with 5% CO_2, the medium/MTT mixtures were removed and the formazan crystals formed by the mitochondrial dehydrogenase activity of vital cells were dissolved in 100 µL of DMSO per well. The absorbance of the wells was read with a microplate reader (Bio-Rad Instruments) at 570 nm. Effects of the drug cell viability were calculated using cell treated with DMSO as control.

Data analysis: Cell survival was calculated using the formula: Survival (%) = [(absorbance of treated cells − absorbance of culture medium)/(absorbance of untreated cells − absorbance of culture medium)] × 100.[45, 46] The experiments were done in triplicate and the inhibitory concentration (IC) values were calculated from a dose response curve. IC_{50} is the concentration in 'µM' required for 50% inhibition of cell growth as compared to that of control. IC_{50} values were determined from the linear portion of the curve by calculating the concentration of agent that reduced absorbance in treated cells, compared to control cells by 50%. Evaluation is based on mean values from three independent experiments, each comprising at least six micro-cultures per concentration level.

Comet assay (Single cell gel electrophoresis):

To assess the genotoxic effect of the new steroidal derivatives, comet assay [47, 48] was performed in MCF7 cells. The MCF7 (1×10^6) cells were treated with 10, 25 and 50 µg/mL of steroidal derivatives for 24 h. The cells were then washed and 200 µL of cell suspension in low melting agarose (LMA) was layered on to the labelled slides precoated with agarose (1.5 %). The slides were placed on ice for 10 min. and submerged in lysis buffer (2.5 % NaCl, 100 mM EDTA, 10 mM Tris, 10 % DMSO and 1% Troton X-100) at 4 °C at pH 10 for more than 1 h. The slides were then equilibrated in alkaline buffer (30 mM NaOH, 1 mM EDTA) at 4 °C at pH 13, electrophoresed at 0.86 V/cm at 4 °C, neutralized, washed and dried. At the time of capturing the images, the slides were stained with ethidium bromide (EtBr, 150 µL 1X) and cover slips were placed over them. For visualization of DNA-damage, EtBr-stained slides

were observed under 209 objectives of a fluorescent microscope (Olympus BX-51, Japan). The images of 50-100 randomly selected cells were captured per slide using a CCD camera.

Treatment of supercoiled plasmid pBR322 DNA with heterosteroids:

To investigate the mechanism of cytotoxicity by studying the effect of heterosteroids on supercoiled plasmid pBR322 DNA, an experiment was done in which the reaction mixture containing 10 mM Tris HCl (pH 7.5), 0.5 μg of pBR322 plasmid DNA, 100 μM copper and the varying concentrations of heterosteroid and Cisplatin (20 μM) were taken. Incubation at room temperature was performed for specified time periods. After incubation, 10 μL of a solution containing 40 mM EDTA, 0.05% bromophenol blue (tracking dye) and 50% glycerol was added and the solution was subjected to electrophoresis in submarine 1% agarose gel. The gel was stained with ethidium bromide (0.5 mg/mL), viewed and photographed on a transilluminator.

Detection of hydroxyl radicals ('OH):

The detection of hydroxyl radicals was investigated by the method studied by Quinlan and Gutteridge.[49] The reaction mixture (0.5 mL) containing Tris HCl (10 mM, pH 7.5), Calf thymus DNA (200 μg), increasing concentrations of heterosteroid and Cisplatin (12.5 μM, 25 μM, 50 μM, 75 μM, 100 μM, 200 μM, 400 μM, 600 μM), Cu (II) (100 μM) and volume was made up to 1 mL by buffer solutions and incubated for 60 min. at 37 °C. Reaction was stopped using 0.5 mL of TCA (28%) and 0.5 mL of 1% TBA was added, boiled for 15 min. and cooled to room temperature. The intensity was read at 532 nm.

Results and discussion

[4', 6'-Dioxo-2'-thioxo-1H-pyrimidin-1-yl]6-imino-5α-cholestane derivatives (94-96)

***In vitro* cytotoxicity:**

The *in vitro* cytotoxicity of steroidal pyrimidines (**94-96**) (refer to **chapter 1** for synthesis) was measured using the MTT assay during which the conversion of the soluble yellowish MTT to the insoluble purple formazan by active mitochondrial lactate dehydrogenase of living cells has been used to develop an assay system for measurement of cell proliferation.[43, 44] The data reported in **Table 15** suggested that compound **94-96** showed different levels of cytotoxicity during which the compound **95** showed effective IC_{50} = 10.39 μmol L^{-1} (HT-29), 10.91 μmol L^{-1} (MCF7), 13.26 μmol L^{-1} (A549). Compound **96** also showed minimum IC_{50} value in the range of 13.04 μmol L^{-1} (HT-29) and 11.41 μmol L^{-1} (MCF7) while as compound **94** showed minimum IC_{50} = 11.72 μmol L^{-1} (A545) and 13.39 μmol L^{-1} (HepG2).

From the **Table 15** it is clear that compound **94** and **95** are showing potential cytotoxicity against A545, A549, HeLa, HepG2 cell lines by showing IC_{50} close to that of Cisplatin, thus can be considered as potential cytotoxic agents. Compound **94-96** also showed better cytotoxicity against HL-60 and HepG2 cell lines by showing IC_{50} less than that of standard anticancer drug, 5-fluorouracil (5-FU).

	IC_{50} (μM)							
Comp.	Lung A545	Breast MCF7	Cervical HeLa	Leukaemia HL-60	Colon SW480	Hepatic HepG2	Colon HT-29	Lung A549
94	11.72	>50	17.12	25.86	37.52	13.39	>50	27.89
95	17.31	10.91	12.37	11.17	>50	19.31	10.39	13.26
96	>50	11.41	27.25	32.34	>50	>50	13.04	>50
Cisplatin	8.9	9.3	9.43	7.83	3.52	9.63	7.24	12.1
5-FU	15.4	14.7	16.32	>50	15.71	>50	9.79	12.8

Table 15. The IC_{50} of compound **94-96**, Cisplatin and 5-FU against given cancer cells

To confirm the toxicity on normal cells, the compounds **94-96** were tested with some non-cancer cell lines; 184B5 (breast), MCF10A (breast), NL-20 (lung), HPC (pulp) and HPLF (Periodontal) during which none of the synthesized compounds were found toxic, all the compounds showed GI_{50} > 60. This also suggests that the steroidal pyrimidine derivatives

can be used specifically for the treatment of cancer cells without showing toxicity to the non-cancer cells. The GI_{50} values shown by compounds **94-96** are given in **Table 16.**

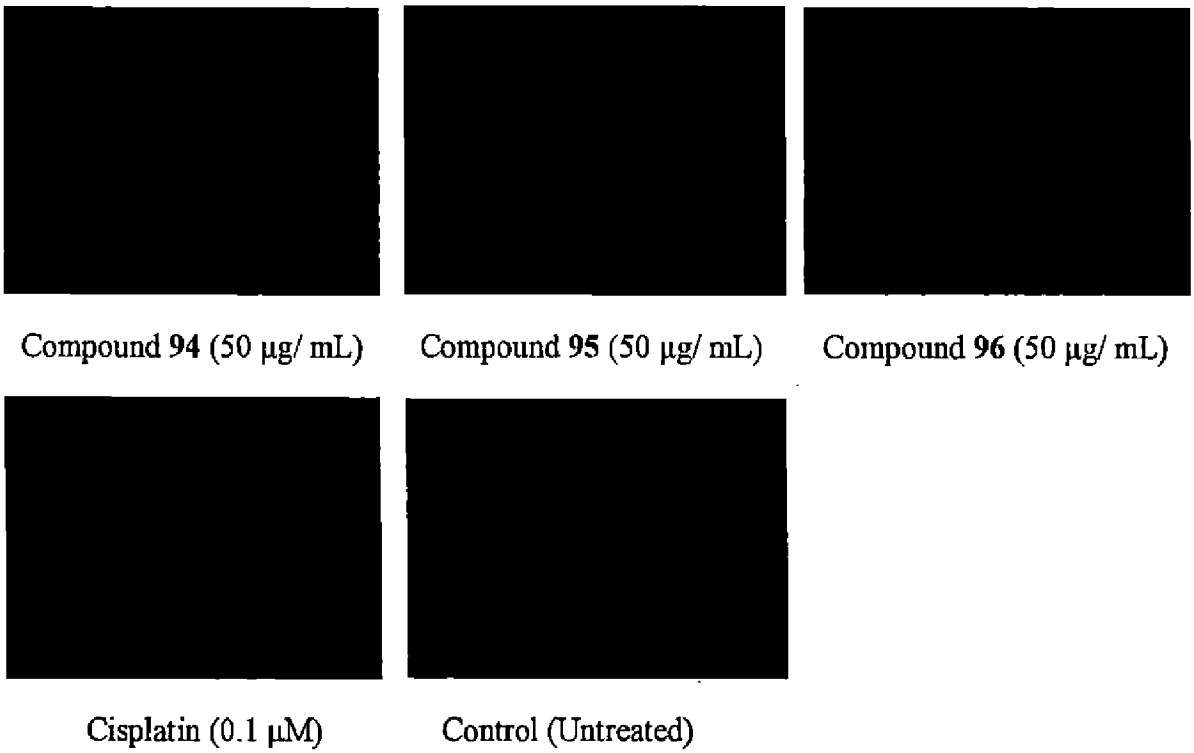
	GI_{50} (μM)				
	Breast	Breast	Lung	Pulp	Periodontal
Compounds	184B5	MCF10A	NL-20	HPC	HPLF
94	74.17	63.62	>100	67.78	67.13
95	78.32	>100	67.25	63.27	>100
96	>100	58.35	61.15	82.12	86.25
Cisplatin	26.17	51.25	63.35	61.17	73.95
5-FU	>100	83.54	73.62	>100	96.51

-GI_{50} is the molar concentration causing 50% growth inhibition of non cancerous cells.
-Values are the mean of triplicates of at least two independent experiments.

Table 16. The GI_{50} of compound **94-96,** Cisplatin and 5-FU against non-cancer cells

Comet assay:

In comet assay, the images of cells treated with Cisplatin and compound **94-96** showed the formation of comets. No comet pattern was observed in the control cells. There was dose-dependent increase in tail length when treated with compound **94-96.** Compound **95** presented maximum apoptotic DNA damage among the three steroidal pyrimidines studied, which was in accordance with its maximum cytotoxicity as seen in MTT assay. None of the steroidal pyrimidines exhibited apoptotic DNA damage to the extent of Cisplatin. The quantified increase in DNA damage suggested that all three pyrimidines derivatives induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for control, cells treated with Cisplatin (0.1 μM, 54 μg/mL), compound **94** (50 μg/mL), compound **95** (50 μg/mL), and compound **96** (50 μg/mL) are shown in **Fig. 2.** Slides were analysed for parameter like tail length (TL), using image analyzer CASP software version 1.2.2. The results of the assay for tail length are shown in **Fig. 3.**



Compound **94** (50 µg/ mL) Compound **95** (50 µg/ mL) Compound **96** (50 µg/ mL)

Cisplatin (0.1 µM) Control (Untreated)

Fig. 2. Detection of DNA damage in MCF7 cells. Treated cells (24 h) were layered over agarose gel, lysed, electrophoresed in alkaline buffer and stained with propidium iodide. Control cells were treated with DMSO alone. The DNA fragmentation resulting in a comet-like appearance in cells treated with Cisplatin and compound **94-96**.

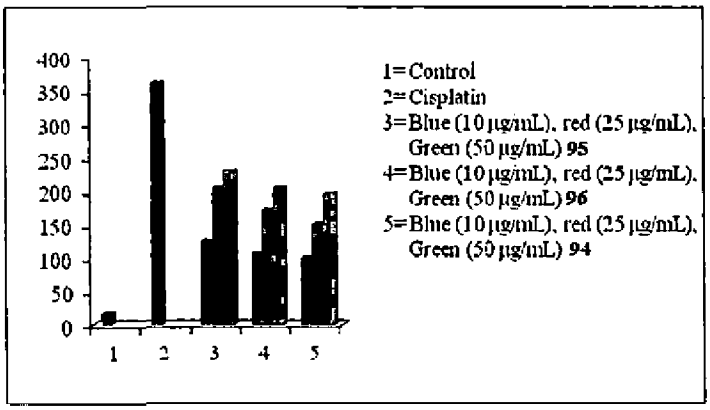


Fig. 3. Comparison of the compound **94-96** and Cisplatin on the tail length in comet assay.

Treatment of supercoiled plasmid pBR322 DNA with compound 95:

Cytotoxicity mechanism was also confirmed by studying the treatment of supercoiled plasmid pBR322 DNA with different concentrations of compound **95** and 100 µM copper. Our nucleolytic experiment suggested that cell death may be due to cleavage or fragmentation of DNA of these cancer cells and that the active species responsible for this are ROS (•OH) which resulted from the *in vitro* reaction of different concentrations of compound

95 with copper in presence of thiobarbituric acid. It has been observed from gel electrophoresis that by adding copper (100 μM) the radical concentration increased which in presence of different concentrations of compound **95** showed the plasmid pBR322 DNA nicking from its supercoiled form (Form I) to open circular form (Form II). As shown in lane 6, 7 and 8 (**Fig. 4**), the nicking is quite obvious by the disappearance of form I and appearance of form II and with the increase in concentration of compound **95** (lane 8), form II became maximum, depicting the more pronounced cleavage at high concentration.

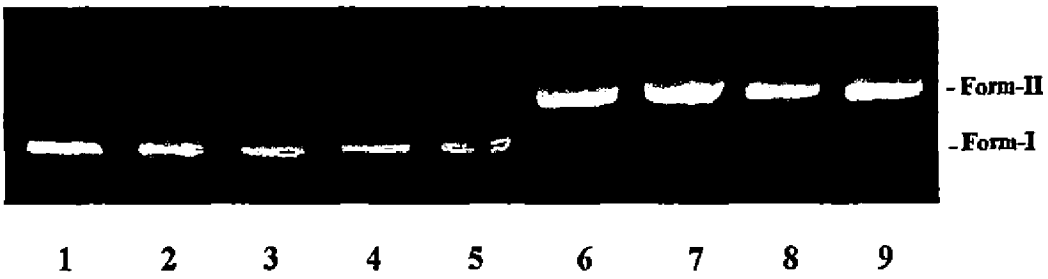


Fig. 4. Fragmentation pattern of supercoiled plasmid pBR322, Lane 1 contains DNA only, lane 2 contains DNA and copper, lane 3, 4 and 5 contain DNA and compound **95** (100, 200 and 300 μM respectively), lane 6, 7 and 8 contain DNA and compound **95** (100, 200, 300 μM respectively) plus 100 μM copper and lane 9 contains DNA, Cisplatin (20 μM) plus 100 μM copper added to it.

Detection of hydroxyl radicals (˙OH):

In the DNA cleavage reactions mediated by various antioxidants in the presence of Cu (II), it has been established that Cu (II) is reduced to Cu (I) by the antioxidants and that Cu (I) is an essential intermediate in the DNA cleavage reactions.[50, 51] It is also generally understood that DNA cleavage by various antioxidants and Cu (II) is the result of the generation of hydroxyl radicals. As mentioned in literature,[52] Cu (II) is reduced to Cu (I) and the re-oxidation of Cu (I) to Cu (II) by molecular oxygen gives rise to superoxide anion which in turn leads to the formation of H_2O_2. Presumably Cu (I) is oxidized to Cu (II) by H_2O_2 in a Fenton type reaction giving rise to hydroxyl radicals.

To determine the hydroxyl radical production and the role of copper ions in DNA cleavage, an experiment was performed where progressively increasing concentrations of compound **95** and Cisplatin (12.5-600 μM) were tested on thiobarbituric acid induced DNA breakage (**Fig. 5**) and from these results we may conclude that the DNA cleavage by thiobarbituric acid involves endogenous copper ions (Cu (I) acts an intermediate) that leads to DNA cleavage.

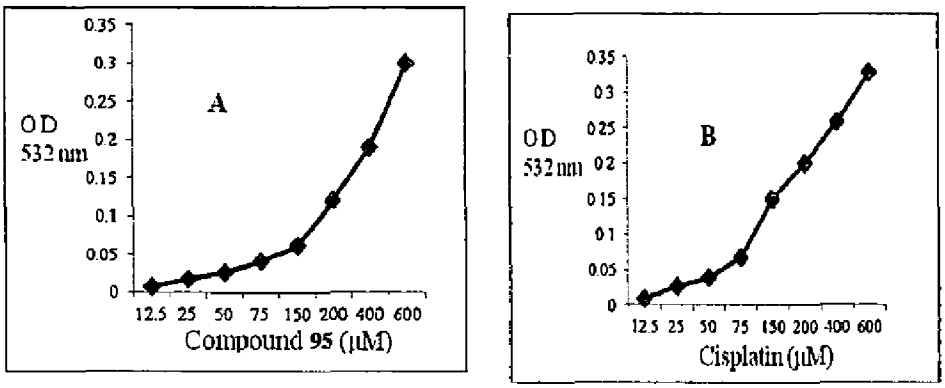


Fig. 5. Determination of hydroxyl radical production by compund **95** (**A**) and Cisplatin (**B**)

The compound **95**-Cu (II) (**Fig. 5A**) and Cisplatin-Cu (II) (**Fig. 5B**) are shown to generate the hydroxyl radicals (˙OH) which react with Calf thymus DNA, resulting in strand breaks. The assay is based on the fact that degredation of DNA by hydroxyl radical results in the release of TBA reactive material, which forms a colored adduct readable at 532 nm.[53] Increasing concentrations of compound **95** or Cisplatin in presence of Cu (II) showed a corresponding increase in the generation of hydroxyl radicals. The results in **Fig. 5** confirmed the relatively higher rate of formation of hydroxyl radicals and correlated with the rate of DNA degredation by the compound **95** as well as Cisplatin.

Work published:

Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Molecular Structure* 1045 (2013) 62-71

[6'-Amino-2'-thioxo-4'-oxodihydro-1H-pyrimidin-1-yl]6-imino-5α-cholestane derivatives (97-99)

In vitro cytotoxicity:

The cytotoxicity of steroidal pyrimidines (**97-99**) (refer to **chapter 1** for synthesis) was also studied by MTT assay.[43, 44] The data reported in **Table 17** suggest that compound **97-99** showed different levels of cytotoxicity and the starting compounds **91-93** were found almost inactive against given cancer cells as they depicted much higher IC_{50}. During the screening the potential behavior was depicted by the compound **97-99** against given cancer cells. The compound **98** showed IC_{50} = 9.63 μmol L^{-1} (HT-29), 9.73 μmol L^{-1} (MCF7), 11.45 μmol L^{-1} (A549). Compound **99** also depicted minimum IC_{50} value in the range of 12.79 μmol L^{-1} (HT-29) and 10.60 μmol L^{-1} (MCF7) while compound **97** showed minimum IC_{50}=9.11 μmol L^{-1} (A545) and 11.26 μmol L^{-1} (HepG2) cell line.

From the **Table 17** it is clear that the IC_{50} for compound **98** against A549 and HeLa cells was found to be 11.45 and 11.72 $\mu M\ L^{-1}$ which is close to the IC_{50} of Cisplatin 12.0 and 9.43 $\mu M\ L^{-1}$, respectively against the same cells.[54] IC_{50} for compound **98** against HeLa cell line was found to be 11.72 which were also close to the IC_{50} of Cisplatin 9.43 against the same cell line.[55] Similarly IC_{50} for compound **97** against HepG2 was found to be 11.26 which is also near to the IC_{50} of Cisplatin 9.8 against the same cell line.[54] It can be concluded that compound **98** and **99** showed potential *in vitro* cytotoxicity against A549, HeLa, HepG2 and A545 cell lines by depicting IC_{50} close to that of Cisplatin, thus can be considered as potential cytotoxic agents. Compound **97-99** also showed marked cytotoxicity against HL-60 and HepG2 cell lines by showing IC_{50} less than standard drug, 5-fluorouracil.

	IC_{50} ($\mu M\ L^{-1}$)							
Comp.	Lung A545	Breast MCF7	cervical HeLa	leukaemia HL-60	Colon SW480	Hepatic HepG2	Colon HT-29	Lung A549
91	18.23	34.58	33.68	>50	29.52	26.72	38.23	29.66
92	29.46	22.17	37.77	45.29	19.26	34.12	26.73	33.18
93	26.28	17.62	39.25	43.21	>50	30.51	35.97	41.32
97	9.11	27.39	15.13	>50	26.11	11.26	17.12	16.31
98	13.45	9.73	11.72	20.17	12.81	11.78	9.63	11.45
99	16.53	10.60	17.37	28.34	>50	24.37	12.79	20.64
Cisplatin	8.9	9.3	9.43	7.83	3.52	9.80	7.24	12.0
5-FU	15.4	15.3	16.32	>50	15.71	>50	9.79	12.8

5-FU = 5-Fluorouracil

Table 17. The IC_{50} of starting compound **91-93** and product **97-99** against cancer cells

To confirm the toxicity on normal cells, the compound **97-99** were tested with some known non-cancer cells; 184B5 (breast), MCF10A (breast), NL-20 (lung), HPC (pulp) and HPLF (Periodontal) during which none of the synthesized compounds were found toxic, all the compounds showed $GI_{50} > 60$. This also suggests that the steroid pyrimidine derivatives can be used specifically for the treatment of cancer cells without being toxic to the normal cells. The GI_{50} values shown by compound **97-99** and Cisplatin are given in **Table 18**.

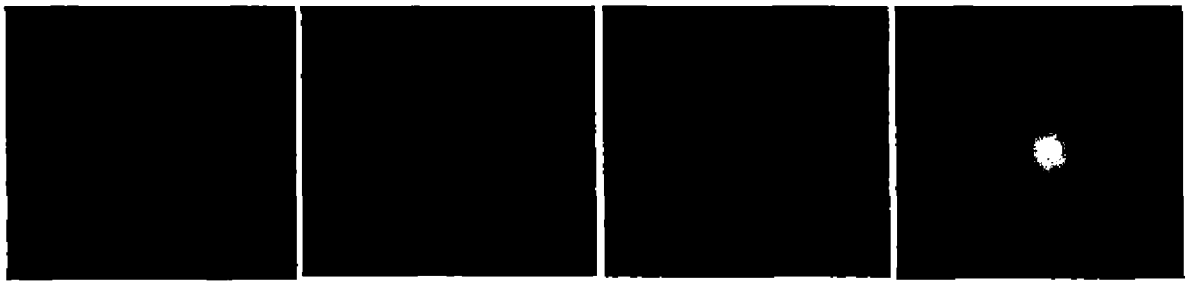
Compounds	GI_{50} (μM) Breast 184B5	Breast MCF10A	Lung NL-20	Pulp HPC	Periodontal HPLF
97	>100	63.62	97.13	64.26	71.13
98	66.13	92.19	85.13	>100	83.25
99	71.25	>100	66.15	73.21	>100
Cisplatin	26.17	51.25	63.35	61.17	73.95
5-FU	>100	83.54	73.62	>100	96.51

-GI_{50} is the molar concentration causing 50% growth inhibition of non-cancerous cells.

Table 18. The GI_{50} of compound **97-99**, Cisplatin and 5-flororuracil against non-cancer cells

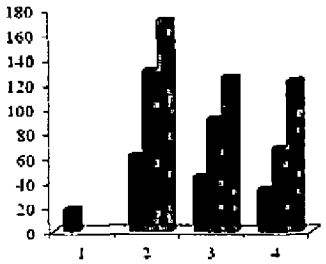
Comet assay:

To understand the apoptotic degradation of DNA, the compound **97-99** were screened against MCF7 in comet assay. There was dose-dependent increase in the tail length when cells were



97 (50 μg/mL) **98** (50 μg/mL) **99** (50 μg/mL) **Control** (Untreated)

Fig. 6. Detection of DNA damage in MCF7 cells. Treated cells (24 h) were layered over agarose gel, lysed, electrophoresed in alkaline buffer and stained with propidium iodide.



1= Control (untreated)
2= Blue (10 μg/mL), red (25 μg/mL), Green (50 μg/mL) **98**
3= Blue (10 μg/mL), red (25 μg/mL), Green (50 μg/mL) **99**
4= Blue (10 μg/mL), red (25 μg/mL), Green (50 μg/mL) **97**

Fig. 7. Comparing the effect of steroidal pyrimidines on the tail length in comet assay treated with compound **97-99**. Compound **98** presented maximum apoptotic DNA damage among the three steroidal pyrimidines studied, which is in accordance with its maximum

cytotoxicity as seen in MTT assay. The quantified increase in DNA damage suggested that the pyrimidine derivative **98** induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for control, cells treated with compound **97-99** (50 µg/mL) are depicted in **Fig. 6**. Slides were analysed for parameter like tail length (TL), using image analyzer CASP software version 1.2.2. The results of the assay for tail length are shown in **Fig. 7**.

Work published;

DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1H-pyrimidines, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Comptes Rendus Chimie*, http://dx. doi.org/10.1016/j.crci.2013.07.001 (in press)

2'-Amino-3'-carboethoxycholest-6-eno [5, 7- *d e*] 4H-pyran derivatives (63-65)

***In vitro* cytotoxicity:**

Literature reveals that synthetic steroids with α, β-unsaturated ketone core gave the potency against human cancer cell lines.[56-59] Thus with this intuition, an attempt of synthesizing the steroidal 4*H*-pyran derivatives (**63-65**) (refer to **chapter 2** for synthesis) from steroidal α, β-unsaturated ketones (**60-62**) was made. Subsequently the compounds were evaluated for anticancer activity towards human cancer cells and in particular, the screening was done with the cell derived from human cancer types: SW480, A549, HepG2, HeLa, MCF7 and HL-60.

The screening data given in **Table 19** shows that compound **63-65** exhibited different levels of cytotoxicity. The preliminary screening showed that some of the compounds were found to have effective IC_{50} i.e. < 20 µmol L^{-1} namely, compound **63** 13.73 µmol L^{-1} (HeLa), 19.29 µmol L^{-1} (A549), 11.18 µmol L^{-1} (MCF7), compound **64** 15.30 µmol L^{-1} (HepG2), 17.12 µmol L^{-1} (MCF7) while compound **65** showed only effective IC_{50} =13.16 against MCF7. From the table it is clear that the IC_{50} for compound **63** against HeLa was found to be 13.73 µmol L^{-1} which is close to the IC_{50} of Doxorubicin 11.53 µmol L^{-1} against the same cell line. Similarly IC_{50} for compound **64** against MCF7 was found to be 11.18 µmol L^{-1} which is more effective than IC_{50} of Doxorubicin 12.41 µmol L^{-1} against the same cell line.

To confirm the toxicity of compounds on non-cancer cells, the compounds **63-65** were tested with two non-cancer cell lines; NL-20 (lung) and HPC (pulp) during which none of the synthesized compounds were found toxic, all the compounds showed IC_{50} > 60 µmol L^{-1}. This also suggests that the steroidal pyran derivatives can be used specifically for the treatment of cancer cells without showing toxicity to the non-cancer cells.

	IC_{50} ($\mu molL^{-1}$)							
	Cancer cells						Non-cancer cells	
Compound	SW480	A549	HepG2	HeLa	MCF7	HL-60	NL20	HPC
63	23.83	19.29	32.43	13.73	11.18	10.37	>100	75.22
64	29.50	22.10	15.30	28.21	17.12	13.17	62.11	87.21
65	31.84	>50	>50	35.91	13.16	28.17	72.18	91.32
Doxorubicin	10.34	8.28	7.36	11.53	12.41	9.64	69.14	81.71
Cisplatin	3.52	12.1	9.63	9.43	9.3	7.83	57.13	64.88

Table 19. Showing IC_{50} of compound **63-65** against cancer and non cancer cells

Comet assay:

In comet assay, the images of cells treated with Cisplatin and compound **63-65** showed the formation of comets but no comet pattern was observed in the control cells. The images of comet assay for control, cells treated with Cisplatin (0.1 μM, 54 μg/mL), compound **63-65** (50 μg/mL) are shown in **Fig. 8** and it is clear that there is dose-dependent increase in tail length when treated with compounds **63-65**. Compound **63** presented highest apoptotic DNA damage

Compound 63 **Compound 64** **Compound 65**

Cisplatin (0.1 μM) **Control** (Untreated)

Fig. 8. DNA damage in MCF7 cells by compound **63-65**. Treated cells (24 h) were layered over agarose gel, lysed and stained with propidium iodide.

among the three steroidal pyrans studied. None of the steroidal pyran exhibited apoptotic DNA damage to the extent of Cisplatin. The quantified increase in DNA damage suggested that all three pyran derivatives induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. Slides were analysed for parameter like tail length (TL), using image analyzer CASP software version 1.2.2. The results of the comet assay for tail length are shown in **Fig. 9** which also suggests that compound **63** showed maximum chromosomal fragmentation at 50 μg/mL leading to apoptosis.

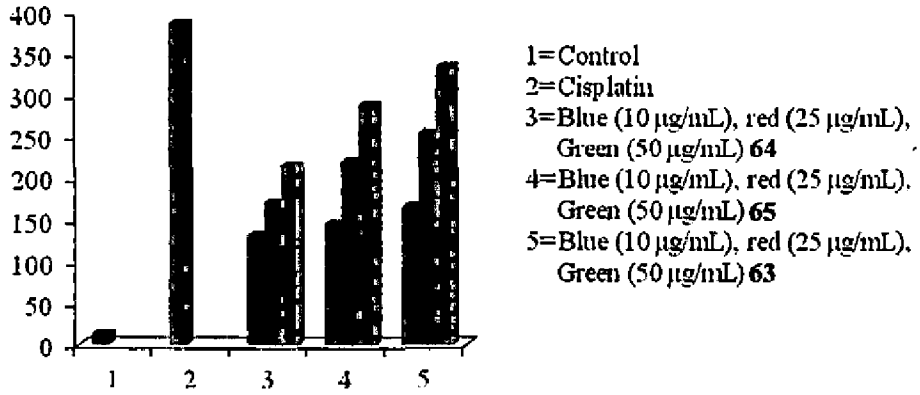


Fig. 9. Comparing the effect of steroidal pyrans on the tail length in comet assay

Treatment of supercoiled plasmid pBR322 DNA with compound 63:

The treatment of supercoiled plasmid pBR322 DNA with different concentrations of compound **63** and 100 μM copper was studied by gel electrophoresis in order to confirm the cytotoxicity mechanism. It was observed from gel electrophoresis that after adding copper (100 μM) the concentration of radicals increase which in presence of different concentrations of compound

Fig. 10. Agarose gel fragmentation pattern of supercoiled plasmid pBR322, Lane 1 contains DNA only, lane 2 contains DNA + copper, lane 3, 4 and 5 contain DNA and compound **63** (100, 200 and 300 μM respectively), lane 6, 7 and 8 contain DNA and compound **63** (100, 200, 300 μM respectively) + 100 μM copper and lane 9 contains DNA, Cisplatin (20 μM) + 100 μM copper added to it.

63 showed the nicking of plasmid pBR322 DNA from its supercoiled form (Form I) to open circular form (Form II). As shown in lane 6, 7 and 8 of the **Fig. 10**, the nicking is quite clear by the appearance of form II and with the increase in concentration of compound **63** (lane 8), form II became maximum, revealing the more pronounced cleavage at high concentration.

Detection of hydroxyl radicals (˙OH):

To study the role of copper ions in DNA cleavage and to determine the hydroxyl radical production, an experiment was performed where progressively increasing concentrations of compound **63** and Cisplatin (12.5-600 μM) were tested on thiobarbituric acid induced DNA breakage. The graphical representation of radical generation in the experiment is shown in **Fig. 11** and from these results we may conclude that the DNA cleavage by thiobarbituric acid involves endogenous copper ions (Cu (I) acts an intermediate) that leads to DNA cleavage.

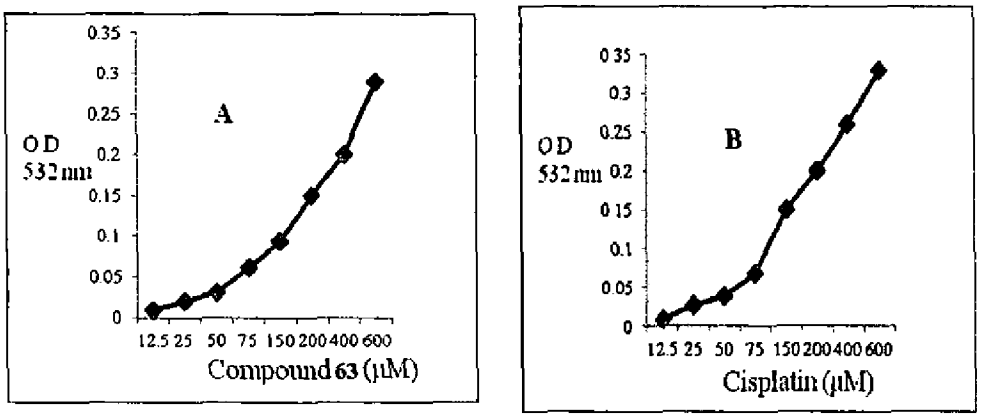


Fig. 11. Determination of hydroxyl radical production by compound **63** and Cisplatin

The compound **63**-Cu (II) **(Fig. 11A)** and Cisplatin-Cu (II) **(Fig. 11B)** are shown to generate the hydroxyl radicals (˙OH) which react with calf thymus DNA, resulting in strand breaks. The assay is based on the fact that degredation of DNA by hydroxyl radical results in the release of TBA reactive material, which forms a colored adduct readable at 532 nm.[53] Increasing concentrations of compound **63** or Cisplatin in presence of Cu (II) showed a corresponding increase in the generation of hydroxyl radicals. The results in **Fig. 11** confirmed the relatively higher rate of formation of hydroxyl radicals and correlated with the rate of DNA degredation by the compound **63** as well as Cisplatin.

Work published;

Synthesis, molecular docking and biological evaluation of new steroidal 4H-pyrans, Shamsuzzaman, **Ayaz Mahmood Dar**, et al, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 117 (2014) 493-501

2'-Amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran derivatives (66-68)

***In vitro* cytotoxicity:**

The steroidal 4H-pyrans **66-68** (refer to **chapter 2** for synthesis) were evaluated for *in vitro* cytotoxicity against cancer cell lines: SW480, A549, HepG2, HeLa, MCF7, DU 145 and HL-60. The preliminary anticancer screening data given in **Table 20** showed that compounds (**66-68**) exhibited different levels of cytotoxicity during which compound **67** is found to have effective IC_{50} (<20 μmol L^{-1}) against given cancer cells; 13.21 μmol L^{-1} (MCF7), 14.77 μmol L^{-1} (DU145), 15.23 μmol L^{-1} (HepG2), 16.56 μmol L^{-1} (SW480) and 19.87 μmol L^{-1} (A549). The compound **68** showed less cytotoxicity as its inhibition count (IC_{50}) against given cancer cells is higher (>20 μmol L^{-1}); 26.46 μmol L^{-1} (A549), 25.72 μmol L^{-1} (HepG2), 36.80 μmol L^{-1} (HeLa) and 33.17 μmol L^{-1} (MCF7). The compound **66** also showed higher IC_{50} against given cancer cells (>20 μmol L^{-1}), 27.45 μmol L^{-1} (SW480), 31.34 μmol L^{-1} (A549), 36.12 μmol L^{-1} (HeLa), 19.72 μmol L^{-1} (MCF7), 28.16 μmol L^{-1} (DU145). The compound **66** was almost inactive against HepG2 and HL-60 cells while compound **68** revealed almost inactive against SW480 due to their IC_{50} >50 μmol L^{-1}.

	IC_{50} (μmol L^{-1})						
	Colon	Lung	Hepatic	Cervical	Breast	Prostate	Leukaemia
Compound	SW480	A549	HepG2	HeLa	MCF7	DU145	HL-60
66	27.45	31.34	>50	36.12	19.72	28.16	>50
67	16.56	19.87	15.23	19.61	13.21	14.77	20.57
68	>50	26.46	25.72	36.80	33.17	41.48	35.61
Doxorubicin	10.34	8.28	7.36	11.53	12.41	6.26	9.64
Cisplatin	3.52	12.1	9.63	9.43	9.3	6.54	7.83

Table 20. Showing anticancer activity data of compound **66-68** against cancer cell lines

From the table it is clear that the compound **67** was found to be potentially cytotoxic among all the three screened compounds and its IC_{50} against MCF7 was found to be 13.21 μmol L^{-1} which is very close to the IC_{50} of standard drug, Doxorubicin (12.41 μmol L^{-1}). Compound **67** also depicted potential cytotoxic behavior against DU145, HepG2 and SW480 cells by showing IC_{50}=14.77 μmol L^{-1}, 15.23 μmol L^{-1} and 16.56 μmol L^{-1}, respectively. Since compound **66** and **68** were not found so much potent as their growth inhibition was at

higher concentration i.e. >25 μmol L^{-1} against given cancer cells except MCF7 cells against which compound **66** expressed a considerable behavior i.e. IC_{50}=19.72 μmol L^{-1}. During anticancer screening, none of the synthesized compounds were found as effective as the standard anticancer drugs like Doxorubicin or Cisplatin, except compound **67** which showed IC_{50}=13.21 μmol L^{-1} which is very close to the IC_{50} of Doxorubicin (12.41 μmol L^{-1}) against MCF7 cell line.

To confirm the cytotoxicity of steroidal pyrans towards normal cells, the compound **66-68** were screened with some non-cancer cells i.e. NL-20 (lung) and HPC (pulp) during which none of the synthesized compounds were found toxic, all the compounds showed GI_{50}>55 μmol L^{-1}. This also suggests that the steroidal pyran derivatives can be used specifically for the treatment of cancer cells. The GI_{50} of compound **66-68**, Doxorubicin and Cisplatin are given in **Table 21**.

	GI_{50} (μML^{-1})		
	Lung	Pulp	Periodontal
Compounds	NL-20	HPC	HPLF
66	>100	>100	>100
67	>100	>100	93.81
68	80.58	>100	>100
Doxorubicin	91.77	>100	>100
Cisplatin	51.25	63.35	61.17

Table 21. The GI_{50} of compound **66-68**, Doxorubicin and Cisplatin against non-cancer cells

Microscopic examination of gross morphology of cancer cells and comparison with steroid pyran **67**-treated cancer cells is shown in **Fig. 12**. The growth of adenocarcinoma colon cells (SW480) treated with 24 mM was inhibited within 24 h (**Fig. B**) but after 37 h cells were completely dead (**Fig. C**). The hepatic carcinoma cells (HepG2) when treated with 24 mM also showed same behavior, the growth was inhibited within 24 h (**Fig. E**) but after 37 h cells were almost completely dead (**Fig. F**). The breast cancer cells (MCF7) and prostate cancer cells (DU145) also followed the same pattern of growth inhibition, as it is clear that after treatment for 24 h with steroidal pyran **67**, the growth is inhibited in MCF7 (**Fig. H**) and DU145 (**Fig. K**) while as after 37 h treatment the MCF7 (**Fig. I**) and DU145 cells (**Fig. L**) were almost completely dead.

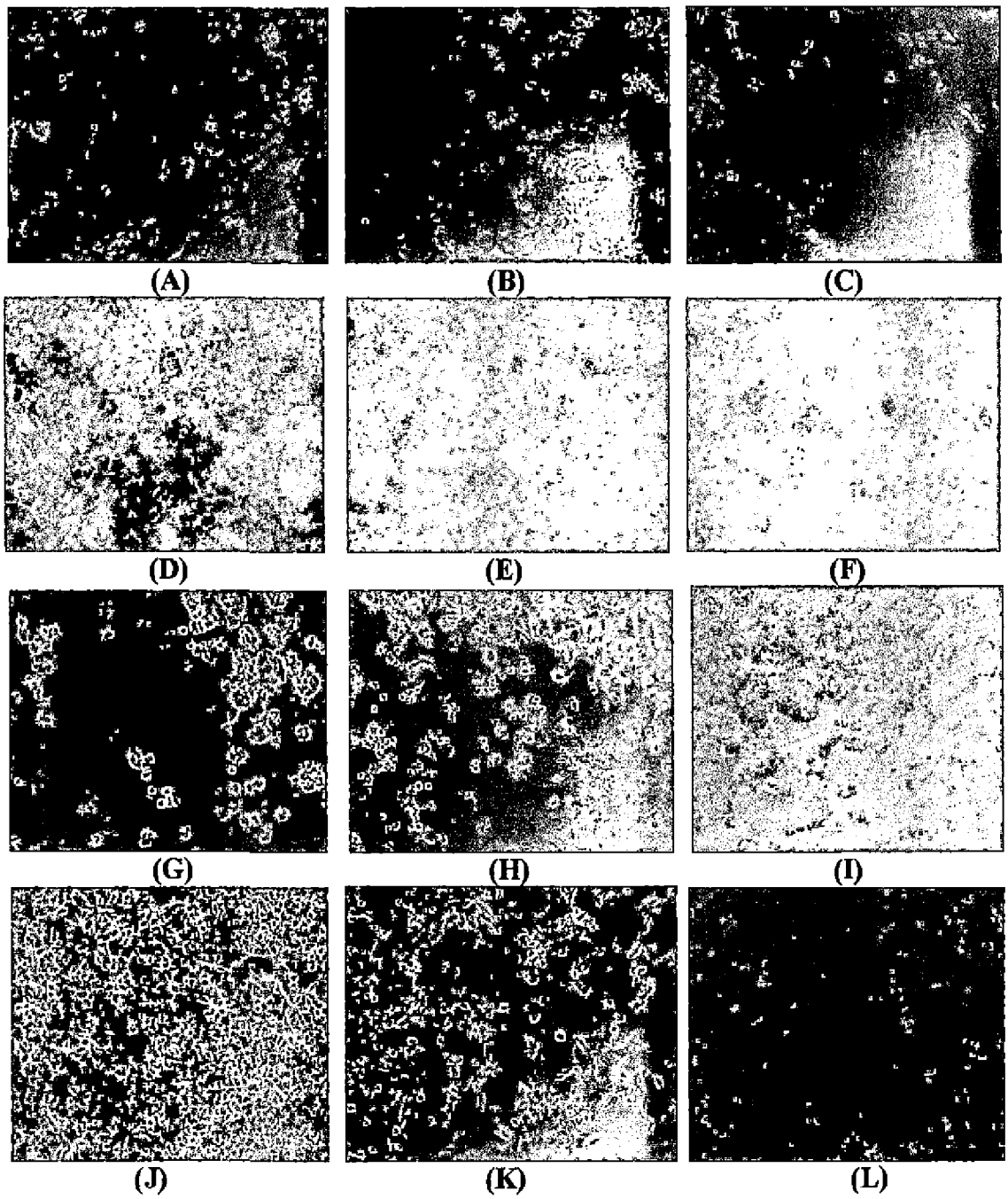


Fig. 12. Microscopic examination of the interaction of cancer cells with steroid pyran **67**. (**A**), (**B**) and (**C**) represent SW480 control and cells treated with 24 mM of steroid pyran **67** for 24 and 37 h, respectively. (**D**), (**E**) and (**F**) represent HepG2 control and cells treated with 24 mM of steroid pyran **67** for 24 and 37 h, respectively. (**G**), (**H**) and (**I**) represent MCF7 control and cells treated with 24 mM of steroid pyran **67** for 24 and 37 h, respectively. (**J**), (**K**) and (**L**) represent DU145 prostatic cancer cell control and cells that reacted with 24 mM of steroid pyran **67** for 24 and 37 h, respectively.

Comet assay (Single cell gel electrophoresis):

To confirm the effect of compounds on DNA damage, cells treated with Cisplatin and compound **66-68** showed the formation of comets. There was dose-dependent increase in tail length when treated with compound **66-68**. Compound **67** presented maximum apoptotic DNA damage among the three steroidal pyrans studied, which was in accordance with its maximum cytotoxicity as seen in MTT assay. The quantified increase in DNA damage suggested that all three pyran derivatives induced dose dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for control, cells treated with compound **66-68** (50 µg/mL), Doxorubicin (0.1 µM) are shown in **Fig. 13**. Slides were analyzed for parameter like tail length (TL), using image analyzer CASP software version 1.2.2. The results of the assay for tail length are shown in **Fig. 14**.

Control (Untreated) **Comp. 66** **Comp. 67** **Comp. 68**

Doxorubicin (50 µg/mL)

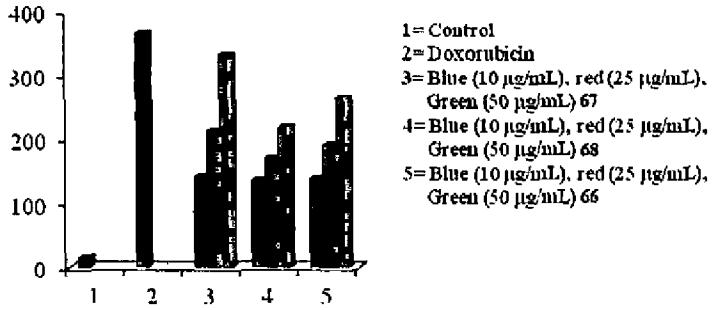


Fig. 14. Comparison of the effect of steroidal pyrans on the tail length in comet assay

Fig. 13. Detection of DNA damage in MCF7 cells. Control cells were treated with DMSO alone. The DNA fragmentation resulting in a comet-like appearance in cells treated with Doxorubicin and compound **66-68**.

Work published;

Synthesis and biological studies of steroidal pyran based derivatives, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Photochemistry and Photobiology B: Biology* 129 (2013) 36-47

5α-Cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole derivatives (67-69)

***In vitro* cytotoxicity:**

The *in vitro* cytotoxicity of steroidal pyrazoles (**67-69**) (refer to **chapter 3** for synthesis) was studied by the MTT assay[43, 44] and the data reported in **Table 22** indicates that they depicted different levels of growth inhibition. The compound **68** showed minimum IC_{50}=16.21 (HL-60), 17.38 (SW480), 17.75 (MCF7), 19.61 (HeLa) while compound **69** revealed effective IC_{50}=14.35 (HL-60), 17.41 (A549), 21.42 (MCF7), 19.27 (HeLa). The inhibitions showed by compound **67** were $\geq$ 20 μmol L^{-1} (as shown in **Table 22**). Thus compound **69** can be considered as potential anticancer agent [IC_{50}=14.35 (HL-60)] among compound **67-69**. The cytotoxicity screening data also suggest that the adjoining of pyrazole scaffold to the steroid nucleus may be one of the factors responsible for the cytotoxicity of the compound **67-69**.

From the **Table 22** it is clear that compound **68** and **69** showed potential cytotoxicity against SW480, HL-60 and MCF7 cell lines by showing IC_{50} close to that of Doxorubicin, thus can be considered as potential cytotoxic agents. Compound **69** even showed IC_{50} same to that of Doxorubicin against HL-60 cell line.

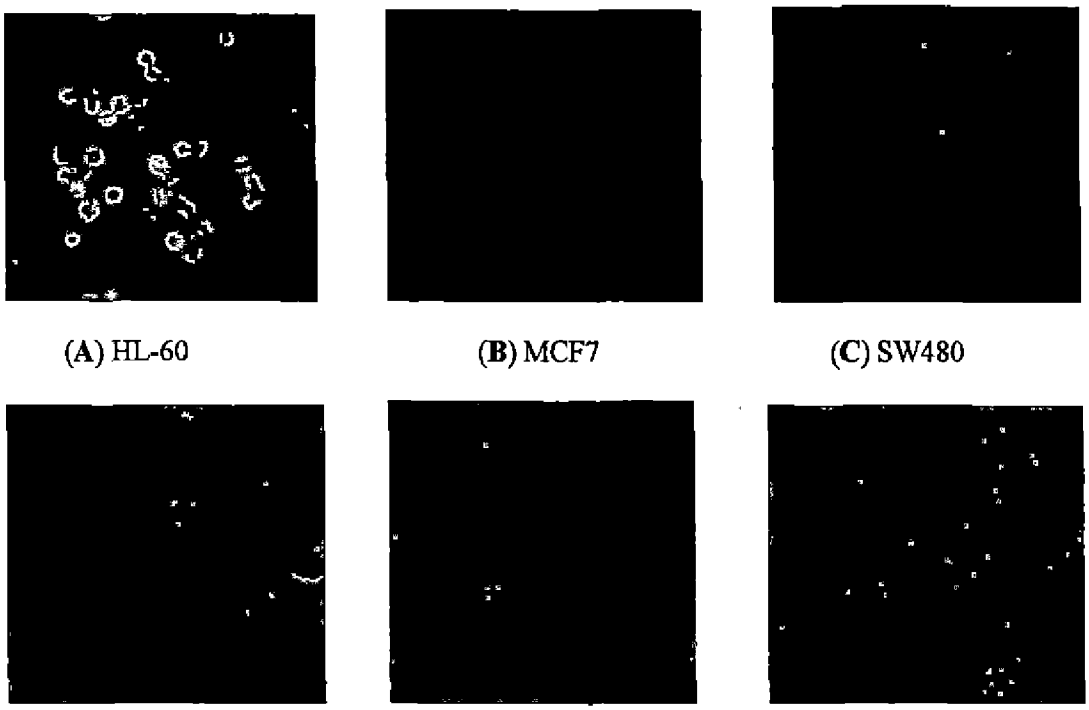
To confirm the toxicity on normal cells, the compound **67-69** were tested with some noncancerous cells i.e. NL-20 (lung) and HPC (pulp) during which none of the synthesized compounds were found toxic, all the compounds showed IC_{50} > 60. This also suggests that these steroidal pyrazoles can be used specifically for the treatment of cancer cells without being toxic to the non-cancer cells.

Compound	IC_{50} (μmol) [a]							
	Cancer cells						Non-cancer cells	
	SW480	A549	HepG2	HeLa	HL60	MCF7	NL20	HPC
67	24.13	20.37	38.12	>50	21.26	25.12	>100	72.15
68	17.38	>50	32.11	19.61	16.21	17.75	64.23	82.11
69	19.52	17.41	22.53	19.27	14.35	21.42	77.12	66.43
Doxorubicin	12.45	9.12	13.21	16.76	14.37	14.21	>100	>100
Cisplatin	3.52	12.1	9.63	9.43	7.83	9.3	63.35	61.17

- [a] IC_{50} is the concentration of compound that inhibits 50% of cell growth

Table 22. Showing IC_{50} of compound **67-69** against Human cancer and non-cancer cells

Microscopic examination of gross morphology of cancer cells and comparison with steroid pyrazole treated cancer cells is shown in **Fig. 15**. The human leukaemia cells (HL-60) when treated with compound **69** (20 mM) showed cytotoxic behavior, their growth was inhibited within 48 h (**Fig. D**). The breast cancer cells (MCF7) followed the same pattern of growth inhibition, as it is clear that after treatment for 48 h with steroidal pyran **69** (20 mM), the growth is inhibited in MCF7 cells (**Fig. E**). The growth of adenocarcinoma cells (SW480) was also greatly inhibited when treated with 20 mM of compound **68** for 48 h (**Fig. F**). The untreated cells (intact nuclei) gave bright green fluorescence whereas treated cells showed sharp orange-red fluorescence showing apoptosis.



(**A**) HL-60 (**B**) MCF7 (**C**) SW480

(**D**) HL-60 + **69** after 48 h (**E**) MCF7 + **69** after 48 h (**F**) SW480 + **68** after 48 h

Fig. 15. Fluorescence microscopic images of untreated (**A**) HL60, (**B**) MCF7 and (**C**) SW480 cells and compound **68** and **69** treated (**D**), HL60, (**E**) MCF7 and (**F**) SW480 cells.

Comet assay:

In the comet assay, the genotoxicity was studied while treating the cells with Cisplatin and compound **67-69** and the formation of the comet is the sign of DNA degradation. There was again dose-dependent increase in tail length when treated with compound **67-69**. Compound **68** presented maximum apoptotic DNA damage among the three steroidal pyrazoles studied, which is in accordance with its maximum cytotoxicity against MCF7 cells as seen in MTT

assay. None of the steroidal pyrazole exhibited apoptotic DNA damage to the extent of Cisplatin. The quantified increase in DNA damage suggested that all three pyrazole derivatives induced dose-dependent fragmentation of chromosomal DNA leading to apoptosis. The images of comet assay for control, cells treated with Cisplatin (0.1 μM, 54 μg/mL), **67**-**69** (50 μg/mL) are shown in **Fig. 16**. Slides were analysed for tail length (TL), as shown in **Fig. 17** using image analyzer CASP software (1.2.2. version).

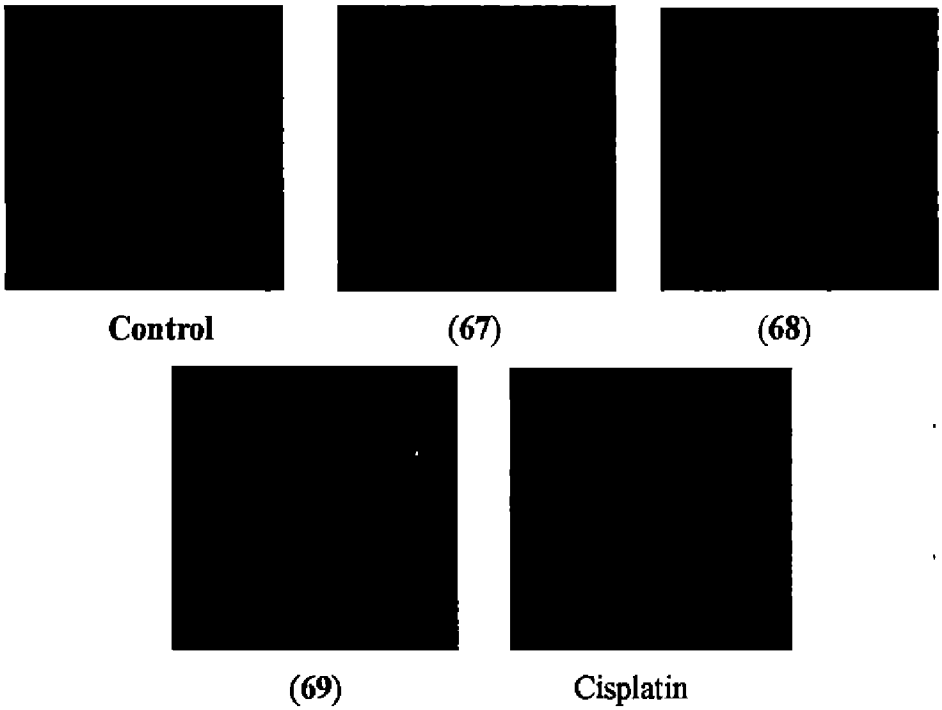


Fig. 16. DNA damage detection in MCF7 cells. Treated cells (24 h) were layered over agarose gel, lysed, electrophoresed in alkaline buffer and stained with propidium iodide.

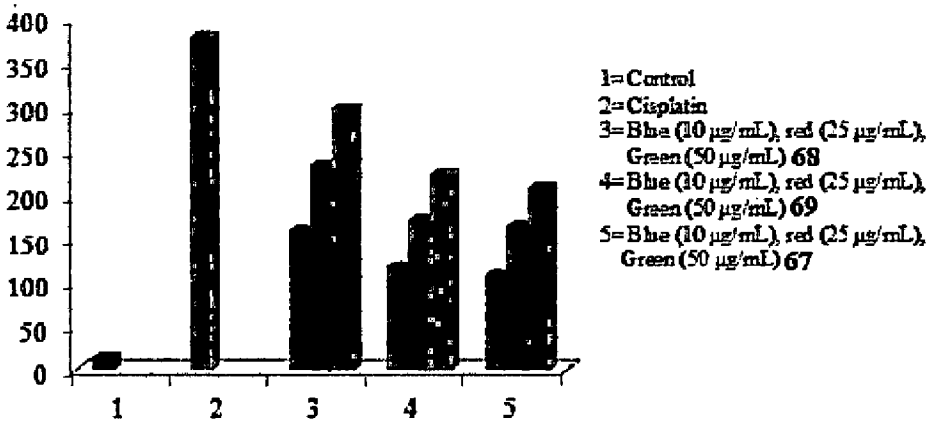


Fig. 17. Graphical representation of the effect of steroidal pyrazoles on DNA damage

5α-Cholestano [5, 6 - *b*] benzothiazine derivatives (53-55)

***In vitro* cytotoxicity:**

The steroidal benzothiazines **53-55** (refer to **chapter 4** for synthesis) were screened for *in vitro* cytotoxicity against human cancer cells; SW480, HeLa, A549 and HepG2 by MTT assay[43, 44] and the screening data reported in **Table 23** suggest that compound **53-55** showed different levels of cytotoxicity during which the compound **53** depicted effective IC_{50} =13.73 μmol L^{-1} against HeLa cells. Compound **54** and **55** also showed minimum IC_{50} value in the range of 15.83 μmol L^{-1} (HepG2) and 16.89 μmol L^{-1} (A549), respectively. Next to these, compound **54** showed minimum IC_{50} =22.67 μmol L^{-1} against SW480 cell line. The IC_{50} shown by the starting ketones (**50-52**) were found either ≥ 40 or much higher >50 μmol L^{-1}. It is clear from the screening data that after the adjoining of benothiazine moiety to the steroidal ketone derivatives (**50-52**), there occurs moderate to good increase in cytotoxicity (**Table 23**). During anticancer screening, only compound **53** showed IC_{50}=13.73 μmol L^{-1} which is more effective than 5-fluorouracil (IC_{50}=16.32 μmol L^{-1}) against HeLa cell line.

Compound	IC_{50} (μmol L^{-1}) [a]			
	SW480	A549	HepG2	HeLa
50	47.28	40.61	>50	>50
51	39.77	46.13	>50	>50
52	>50	>50	49.22	>50
53	27.73	16.89	>50	13.73
54	22.67	29.85	15.83	31.43
55	>50	41.52	37.65	49.18
Doxorubicin	10.9	13.5	11.52	12.52
5-Fluorouracil	15.71	12.8	33.6	16.32
Cisplatin	3.52	12.1	9.63	9.43

Table 23. IC_{50} of starting compounds **50-52** and products **53-55** against human cancer cells

To confirm the cytotoxicity of steroidal benzothiazines on normal cells, the compound **53-55** were tested with some non-cancer cells i.e. MCF10A (breast), NL-20 (lung), HPC (pulp) and HPLF (Periodontal) during which none of the synthesized compounds were found toxic, all the compounds showed GI_{50} > 55 μmol L^{-1}. The GI_{50} of compound **53-55** and Cisplatin, Doxorubicin and 5-fluorouracil are given in **Table 24**.

	GI_{50} (μML^{-1})			
	Breast	Lung	Pulp	Periodontal
Compounds	MCF10A	NL-20	HPC	HPLF
53	>100	60.92	67.78	>100
54	68.41	90.21	63.31	>100
55	72.11	>100	59.16	66.33
Doxorubicin	>100	91.77	>100	>100
Cisplatin	26.17	51.25	63.35	61.17
5-FU	>100	83.54	73.62	>100

Table 24. The GI_{50} of compound **53-55**, Doxorubicin, Cisplatin and 5-fluorouracil against non-cancerous cells

Work published;

Anticancer and antimicrobial evaluation of newly synthesized steroidal 5, 6 fused benzothiazines. Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Arabian Journal of Chemistry*, http://dx.doi.org/10.1016/j.arabjc.2013.06.027, in press

2'-Hydrazinocholest-6-eno [4, 5 - *d*] thiazole derivatives (56-58)

***In vitro* anticancer activity:**

The inhibitory effect of steroidal thiazoles **56-58** (refer to **chapter 4** for synthesis) on human cancer cells was measured by MTT assay.[43, 44] The results are expressed as IC_{50} (**Table 25**) which depict that compound **56-58** showed different levels of activity.

Compound	IC_{50} (μmol L^{-1})				
	SW480	A549	HepG2	HeLa	HL-60
56	14.03 ± 0.2	13.22 ± 0.7	17.37 ± 1.5	16.62 ± 0.4	>50
57	13.04 ± 0.6	11.32 ± 0.2	9.71 ± 1.1	13.17 ± 0.4	14.71 ± 0.3
58	21.66 ± 0.4	15.44 ± 1.3	>50	11.74 ± 0.7	26.27 ± 0.5
Cisplatin	3.52 ± 0.3	10.51 ± 0.2	9.6 ± 0.9	9.43 ± 0.5	7.8 ± 1.5

Table 25. Showing the IC_{50} of compound **56-58** against given human cancer cell lines

The compound **57** showed minimum IC_{50}= 9.71 ± 1.1 (HepG2), 11.32 ± 0.2 (A549), 13.04 ± 0.6 (SW480) and 13.17 ± 0.4 μmol L^{-1} (HeLa) while compound **58** showed minimum IC_{50}=11.74 ± 0.7 (HeLa) and 15.44 ± 1.3 μmol L^{-1} (A549). The minimum inhibitions shown by compound **56** were 13.22 ± 0.7 (A549), 14.03 ± 0.2 (SW480) and 16.62 ± 0.4 μmol L^{-1} (HeLa).

From these results it is clear that the IC_{50} for compound **57** against A549 cell line is 11.32 ± 0.2 which is very close to the IC_{50} of Cisplatin (10.51 ± 0.2) against the same cell line. IC_{50} for compound **58** against HeLa cell line is 11.74 ± 0.7 which is also close to the IC_{50} of Cisplatin (9.43 ± 0.5) against the same cell line. Similarly IC_{50} for compound **57** against HepG2 is 9.71 ± 1.1 which is also near to the IC_{50} of Cisplatin (9.6 ± 0.3) against the same cell line. It can be concluded that all the compounds (**56-58**) showed potential anticancer activity against A549, HepG2, HeLa cell lines by depicting IC_{50} close to that of standard drug, Cisplatin thus can be considered as potential anticancer agents.

Treatment of supercoiled plasmid pBR322 DNA with compound 57:

It has been observed from gel electrophoresis that after adding copper (100 μM), the concentration of radicals increase which in presence of different concentrations of compound **57** showed the nicking of plasmid pBR322 DNA from its supercoiled form (Form I) to open circular form (Form II). As shown in **Fig. 18**, lane 6, 7 and 8, the nicking is quite obvious by the appearance of form II and with the increase in concentration of compound **57** (lane 8) the band intensity (form II) became maximum, depicting the more pronounced cleavage at high concentration.

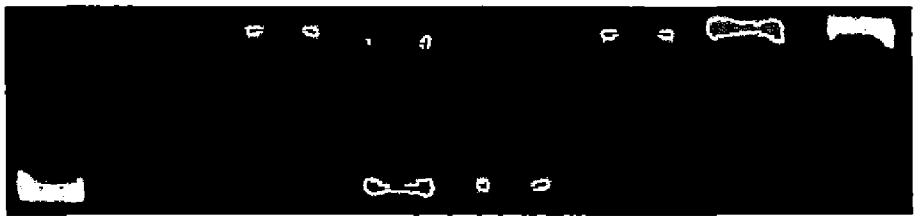


Fig. 18. Fragmentation pattern of supercoiled plasmid pBR322, Lane 1 contains DNA only, lane 2 contains DNA and copper, lane 3 and 4 contain DNA and compound **57** (200 and 300 μM respectively), Lane 5, 6 and 7 contain DNA and compound **57** (200, 300, 400 μM respectively) + 100 μM copper added to it and Lane 8 contains DNA+ Cisplatin (20 μM) + 100 μM copper added to it.

Detection of hydroxyl radicals (OH):

To determine the hydroxyl radical production and the role of copper ions in DNA cleavage, an experiment was performed where progressively increasing concentrations of compound **57** and Cisplatin (12.5-600 μM) were tested on thiobarbituric acid induced DNA breakage (**Fig. 19**) and from these results we may conclude that the DNA cleavage by thiobarbituric acid involves endogenous copper ions and also that Cu (I) is an intermediate in the pathway that leads to DNA cleavage.

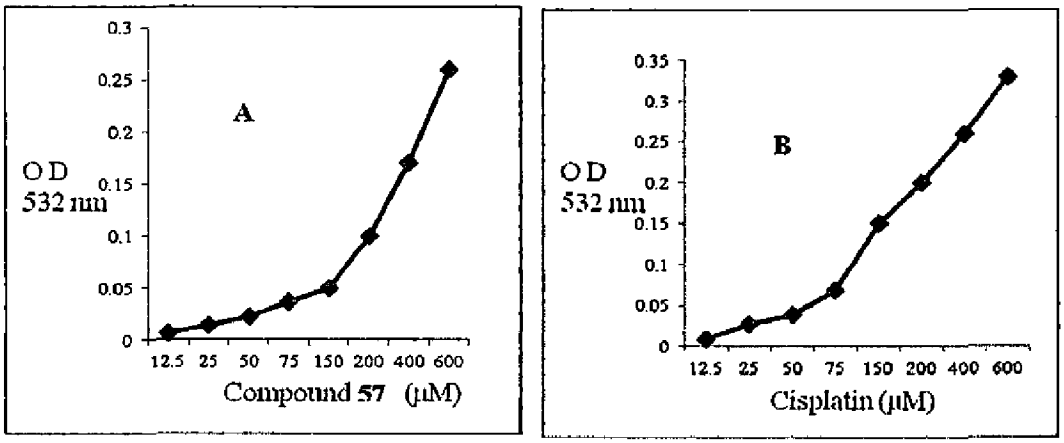


Fig. 19: Showing comperative determination of hydroxyl radical production by compund **57** (**A**) and Cisplatin (**B**) by the assay of Thiobarbituric acid

The compound **57**-Cu (II) **(Fig. 19A)** and Cisplatin-Cu (II) **(Fig. 19B)** are shown to generate the hydroxyl radicals (OH) that react with CT DNA, result in strand breaks. The assay is based on the fact that degredation of DNA by hydroxyl radical results in the release of TBA reactive material, which forms a colored adduct readable at 532 nm.[53] Increasing concentrations of compound **57** or Cisplatin in presence of Cu (II) showed a corresponding increase in the generation of hydroxyl radicals. However the generation of hydroxyl radical being more in case of Cisplatin as shown in **Fig. 19(B)**. The results in **Fig. 19** confirmed the relatively higher rate of formation of hydroxyl radicals and correlated with the rate of DNA degredation by the compound **57** as well as Cisplatin.

Work accepted;

Synthesis, characterization and in vitro anticancer activity of newly synthesized steroidal 6, 7-fused thiazoles, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Chemistry* (accepted)

Antioxidant activity

Experimental

2, 2-Diphenyl-2-picryl-hydrazyl (DPPH) assay:

The 2, 2-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity of the heterosteroids was measured according to the literature method.[60] Drug stock solution (1 mg/mL) was diluted to final concentration of 2, 4, 6, 8, 10 and 12 in methanol. Methanolic DPPH solution (1 mL, 0.3 mmol) was added to 3.0 mL of drug solution of different concentrations. The tube was kept at an ambient temperature for 30 min and the absorbance was measured at 517 nm. The scavenging activity was calculated by following formula: [% inhibition = $[(A_{Control} - A_{Sample}/A_{Control} \times 100]$ Where $A_{Control}$ is the absorbance of the ascorbic acid (standard) and A_{Sample} is the absorbance of different compounds. The methanolic DPPH solution (1 mL, 0.3 mM) was used as control.

Hydrogen peroxide (H_2O_2) scavenging assay:

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. There is increasing evidence that hydrogen peroxide, either directly or indirectly via its reduction product hydroxyl radical (˙OH) causes severe damage to biological systems. In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm.[61] The method was carried out with minor modifications. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 mL of the steroidal compounds or standards in methanol were added to 2 mL of hydrogen peroxide solution in PBS. The absorbance was measured at 230 nm after 10 min.

Scavenging of nitric oxide (SNP) radical assay:

Aqueous solution of sodium nitroprusside (SNP) at physiological pH spontaneously generates nitric oxide (NO) which interacts with oxygen to produce nitrite ions that can be estimated by the use of modified Griess Ilosvay reaction with little modifications. Scavengers of NO compete with oxygen leading to reduce production of NO. The reaction mixture (6 mL) containing SNP (10 mM, 4 mL), phosphate buffer saline (PBS, pH 7.4, 1 mL) and steroidal compounds or standard (1 mL) in DMSO at various concentrations was incubated at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture containing nitrite ion was removed, 1 mL of sulphanilic acid reagent was added, mixed well and allowed to stand for 5 min. for completion of diazotization. Then 1 mL of NEDD was added, mixed and allowed to stand for 30 min. in diffused light. A pink colored chromophore was formed. The absorbance was measured at 540 nm.[62]

Results and discussion

[4', 6'-Dioxo-2'-thioxo-1H-pyrimidin-1-yl]6-imino-5α-cholestane derivatives (94-96)

Many thiosemicarbazone derivatives with a broad spectrum of effects are known to act as free radical inhibitors. [63] The DPPH assay[60] is widely used for assessing the ability of radical scavenging activity of these compounds. Because of the presence of odd electron, DPPH shows a strong absorption band at 517 nm in the visible spectrum. As this electron becomes paired off in the presence of a free radical scavenger, this absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. The result of DPPH assay of the steroidal pyrimidine (**94-96**) (refer to **chapter 1** for synthesis) is shown in **Table 26** and it is clear that all the compounds exhibited potential antioxidant activity compared to the standard, ascorbic acid. Compound **96** showed maximum antioxidant activity by depicting the higher absorbance among all the three screened compounds.

Compound	Absorbance value ± S.D.			
	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
94	17.8±0.2	26.4±0.4	31.6±0.2	37.5±0.3
95	21.2±0.5	29.3±0.2	32.4±0.4	38.4±0.2
96	26.2±0.3	30.2±0.5	35.7±0.4	39.8±0.6
Ascorbic acid	35.0±0.4	40.0±0.2	39.0±0.3	46.0±0.5
Control	-	-	-	-

-Values represent the mean ± standard error mean (SEM) of three experiments.

Table 26. Showing reducing power of steroidal pyrimidines (**94-96**)

2'-Amino-3'-carboethoxycholest-6-eno [5, 7 - *d e*] 4H-pyran derivatives (63-65)

Many pyran derivatives with a broad spectrum of biological activity are known to show free radical scavenging activity. One of the pyran derivatives, kojic acid is known to have potential antioxidant activity. With this intrest, the steroidal pyrans (**63-65**) were screened for DPPH scavenging assay and the results are given in **Table 27**. But during the antioxidant screening by DPPH assay[60], it was found that steroidal pyrans (**63-65**) (refer to **chapter 2** for synthesis) were not significantly active compared to the standard, ascorbic acid. The antioxidant activity shown by the steroidal pyrans was found to be less than 50% of the ascorbic acid.

Compound	Absorbance value ± S.D.			
	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
63	9.3±0.2	12.5±0.4	13.2±0.6	17.1±0.3
64	11.2±0.4	13.6±0.2	16.2±0.4	16.9±0.3
65	10.2±0.3	11.8±0.4	15.2±0.1	19.8±0.6
Ascorbic acid	35.0±0.4	40.0±0.2	39.0±0.3	46.0±0.5
Control	-	-	-	-

-Values represent the mean ± standard error mean (SEM) of three experiments.

Table 27. Showing reducing power of steroidal pyrans **(63-65)**

Cholestano [6, 7 - *c*] 5'-methyl-1'-carbothioic acid amido pyrazole derivatives (67-69)

Since thiosemicarbazones have often depicted the free radical inhibition in a potential manner.[63] Thus, pyrazole which is the derivative of thiosemicarbazone has also got tendency to act as the effective radical scavenger. Hence the H_2O_2 scavenging assay[61] of steroidal pyrazoles (**67-69**) (refer to **chapter 3** for synthesis) has been done and the results are shown in **Table 28**. It is clear that the compound **67-69** were found significantly active in antioxidant activity compared to the standard, ascorbic acid.

Compound	Absorbance value ± S.D.			
	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
67	19.3±0.5	27.4±0.1	33.1±0.3	36.2±0.1
68	21.5±0.1	28.3±0.6	31.6±0.2	38.3±0.6
69	18.7±0.2	26.8±0.4	32.4±0.6	40.1±0.3
Ascorbic acid	35.0±0.4	40.0±0.2	39.0±0.3	46.0±0.5
Control	-	-	-	-

-Values represent the mean ± standard error mean (SEM) of three experiments.

Table 28. Showing reducing power of steroidal pyrazoles **(67-69)**

2′-Hydrazinocholest-6-eno [4, 5 - *d*] thiazole derivatives (56-58)

Among sulfur compounds, thiazole is an important compound with wide number of applications. Thus, as per literature, the thiazoles can act as the effective radical scavengers. Thus the SNP radical scavenging assay[62] of steroidal thiazoles (**56-58**) (refer to **chapter 4** for synthesis) has been done and the results are shown in **Table 29**. It is clear that the compounds (**56-58**) were found significantly active in antioxidant activity compared to the standard, ascorbic acid.

Compound	Absorbance value ± S.D.			
	25 µg/mL	50 µg/mL	75 µg/mL	100 µg/mL
56	19.6±0.4	26.4±0.3	30.1±0.2	37.4±0.2
57	20.2±0.3	29.3±0.2	34.6±0.5	39.8±0.6
58	16.7±0.2	22.2±0.1	27.4±0.5	33.5±0.6
Ascorbic acid	35.0±0.4	40.0±0.2	39.0±0.3	46.0±0.5
Control	-	-	-	-

-Values represent the mean ± standard error mean (SEM) of three experiments.

Table 29. Showing reducing power of steroidal thiazoles (**56-58**)

DNA binding studies

DNA is an important template as it regulates many biochemical processes that occur in the cellular system. The different loci present in the DNA are involved in various regulatory processes such as gene expression, gene transcription, mutagenesis, carcinogenesis, etc.[64] Many synthetic molecules exert their anticancer activities by binding with DNA, thereby altering DNA replication and inhibiting the growth of tumor cells. DNA cleavage reaction is also considered of prime importance as it proceeds by targeting various constituents of DNA *viz.*, the nucleic bases, deoxyribose moiety and phosphodiester linkage. Therefore, synthetic molecules that undergo hydrolytic DNA cleavage are useful in genetic engineering, molecular biotechnology and robust anticancer drug design.[65, 66]

In the field of molecular biology and drug development, the cleaving agents of nucleic acid have attracted extensive attention due to their potential applications.[67] Under uncatalyzed physiological conditions, the phosphodiester bonds of DNA are extremely stable and the half life of DNA for hydrolysis is estimated to be around 200 million years.[68] Metal complexes have been widely investigated as cleaving agents of nucleic acids and are found to be reasonably efficient,[69] but their use in pharmacy is restricted because of serious issues over the lability and toxicity, that limits the practical usage of these compounds.[70] To overcome these limitations, Gobel and co-workers[71] put forward the concept of 'metal-free cleaving agents' which are being applied to active phosphodiesters like nucleic acid mimic and RNA.

Since DNA has been identified as the primary molecular target of heterocyclic based anticancer drugs, interaction of well tailored heterocycles with DNA ascertains the extent and mode of DNA binding and the potential of these compounds to act as chemotherapeutic agents. There are various modes of DNA interaction; covalent, non-covalent, intercalation etc. DNA targeted metal based drugs which bind to nucleobase moieties has shown low degree of selectivity and high toxicity to the normal cells. Recently, non-covalent DNA binding organic heterocycles particularly pyrimidine derivatives have received attention in the development of efficient anticancer drugs (5-fluorouracil).[72] Intercalation and electrostatic interactions are well known effects that lead to the hypochromism and hyperchromism shifts, respectively in the DNA.

Experimental

Material and equipments:

Super coiled pBR322 DNA was purchased from GeNei (India) and was used for the agarose gel experiment without further purification. Calf thymus DNA (CT DNA) and pUC 19 DNA were purchased from Sigma, were dissolved in a 0.1 M Tris-buffer. The purity of DNA was verified by monitoring the ratio of absorbance at 260 nm to that of 280 nm, which was in the range 1.8-1.9. The concentration of the DNA was determined spectrophotometrically using ε_{260} = 6600 M^{-1} cm^{-1}. T4 DNA ligase enzyme was purchased from CalBioChem and was utilized as received. The UV-Vissible measurements were recorded on a Beckman DU 40 Spectrophotometer (USA) while as fluorescence measurements were recorded on a Shimadzu Spectrofluorimeter-5000 (Japan). Cleavage experiments were performed with the help of Axygen electrophoresis supported by Genei power supply with a potential range of 50-500 V, visualized and photographed by Vilber-INFINITY gel documentation system.

Gel electrophoresis:

Two concentrations (100 and 200 μM) of compounds and 3μL of 0.5 μg/μL of pUC19 DNA was loaded with 2 μL of EtBr into 1% agarose gel. The final volume of the reaction mixture for all the wells of gel was 10 μL. The DNA band was visualized under UV transilluminator.

Absorption spectroscopy:

The UV-Vissible measurements of calf thymus DNA were recorded on a Beckman DU 40 Spectrophotometer (USA) by using a cuvette of 1cm path length. The absorbance values of compounds in the absence and presence of DNA were recorded in the range of 225-350 nm. Appropriate blanks corresponding to the DNA solutions and buffer were subtracted to correct the base line.

Fluorescence spectroscopy:

Fluorescence measurements were recorded on a Shimadzu Spectrofluorimeter-5000 (Japan). The fluorescence quenching with increasing concentration of DNA was recorded after exciting the drug solution at 280 nm, using 10/10 nm as slit widths. After subtracting the value of the baseline (buffer alone), the uncorrected fluorescence values, F^{UC}, for compounds was corrected as follows

$F = F^{UC} \times (3000 + x) / 3000$

where 'x' is the volume of the added DNA solution (in microliters), 3000 is a mixture volume (in microliters), before the addition of DNA, and *F* is a corrected fluorescence value.[73, 74]

Molecular Docking:

The rigid molecular docking studies were performed using HEX 6.1 software.[75] The initial structure of the compounds was generated by Mercury modeling software. The molecules of compounds were optimized for use in the following docking study. The crystal structure of the B-DNA dodecamer d(CGCAAATTTCGC)$_2$ (PDB ID: 1BNA) was downloaded from the protein data bank. All calculations were carried out on an Intel CORE i5, 2.6 GHz based machine running MS Windows 7 as the operating system. Visualization of the docked pose have been done using PyMol molecular graphics program.[76]

Nuclease or cleavage activity:

Cleavage experiments of supercoiled pBR322 DNA (300 ng) by new heterosteroids (1.0-5.0 μM) in (5 mM Tris-HCl/50 mM NaCl), buffer at pH 7.2 were carried out and the reaction followed by agarose gel electrophoresis. The sample was incubated for 1 h at 37 °C. A loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol was added and electrophoresis was carried out at 60 V for 1 h in Tris-HCl buffer using 1% agarose gel containing 1.0 μg/mL EtBr. The reaction was also monitored upon addition of various radical inhibitors and/or activators such as DMSO, *tert*-butyl alcohol (TBA), sodium azide (NaN_3), superoxide dismutase (SOD), mercaptopropionic acid (MPA), glutathione (GSH) and H_2O_2.

T4 ligation experiment:

To support the hydrolytic mechanism of DNA cleavage, the DNA relegation experiments were performed using T4 ligase enzyme. The compounds treated with pBR322 plasmid DNA (2 mg), ligation buffer of 1.5 mL in 10X, T4 ligase 1 mL (2 units) and 2.5 mL of H_2O were mixed and incubated at 4 °C for 1 h. Subsequently the samples were loaded on 1% agarose gel and visualized by staining with an ethidium bromide solution.

Results and discussion

DNA binding studies of [4', 6'-dioxo-2'-thioxo-1H-pyrimidin-1-yl] 6-imino-5α-cholestane derivatives (94-96)

Gel electrophoresis: The steroidal pyrimidines (**94-96**) (refer to **chapter 1** for synthesis) were examined for their binding abilities with pUC19 DNA by agarose gel electrophoresis. In the **Fig. 20**, lane 1 contains DNA only, lane 2, 4, 6 contains DNA and 100 μM of compound **94-96** and lane 3, 5, 7 contain DNA and 200 μM of compound **94-96**. At low concentration, the compound **94-96** do not show any appreciable change in the band intensity of DNA (lane 2, 4, 6). However, at higher concentrations band intensity gets diminished in compound **94-96** (lane 3, 5, 7). Thus electrophoretic pattern demonstrate that all the compounds interact with DNA. The loss in the band intensity at higher concentration may be assumed due to the intercalation of the compound **94-96** to DNA, which in turn results in the displacement of EtBr. In case of lane 2, 4, 6 the concentration of the compounds is not sufficient for the displacement of EtBr. This conclusion was further supported from the fluorescence quenching, which gave convincing evidence for interaction of the compounds with DNA.

UV-vis absorption spectroscopy: Absorption spectra of the compound **94-96** showed quite similar broad, unstructured lowest-energy bands with maxima in the vicinity of 285 nm. **Fig. 21** shows the absorption spectra of the compounds in presence of various concentrations of CT DNA. The concentrations of all the compounds were maintained at 100 μM to make an easier comprehension and comparison of the extent of binding interaction of the compound **94-96** to DNA. It was found that the absorption intensity in the compound **94-96** increased with gradual addition of DNA that showed hyperchromism, with slight bathochromic shifts. Such type of bathochromic shift suggests an interaction between DNA and compound **94-96**. An interaction between compound **94-96** and DNA decreases the energy gap between the LUMO and HOMO of the compound **94-96** that results in a bathochromic shift. As can be seen from the **Fig. 21** the isobestic point is not clear, which hinders in calculating the binding constant values, suggests that DNA and compound **94-96** does not bind in 1:1 ratio or there may be more than one mode of binding.

Fluorescence spectroscopy: To obtain an insight into the binding of compound **94-96** with DNA, the fluorescence quenching of compound **94-96** was studied using DNA as a quencher, and the degree of the accessibility of each of the molecule to the quencher was examined. **Fig. 22** depicts change in the emission spectrum of these compounds in presence of DNA.

The fluorescence quenching data was analyzed to obtain the quenching constant by using the well-known Stern-Volmer equation (1).[77]

$$\left(\frac{F_o}{F}\right) - 1 = k_{sv}[Q] \qquad (1)$$

Where F_o and F denote the steady-state fluorescence intensities in the absence and in the presence of quencher (DNA), respectively, K_{SV} is the Stern-Volmer quenching constant (which is a measure of quenching efficiency), and [Q] is the concentration of the quencher. Hence, eq. 1 was applied to determine K_{SV} by linear regression of a plot of $(F_o/F)-1$ versus concentration of DNA and is represented in **Fig. 22.** The Stern-Volmer constants (K_{SV}) estimated for compound **94-96** by Stern-Volmer equation were recorded in **Table 30.** The Stern-Volmer plot is linear, indicating that only one type of quenching process occurs, either static or dynamic.

Compounds	$K_{SV}(1\times10^{-3})$ (M^{-1})	K_q (1×10^{-11}) $(M^{-1}s^{-1})$	$K(1\times10^{-3})$ (M^{-1})	n	r^2
94	1.54	1.54	4.634	1.124	0.9937
95	2.44	2.44	2.123	0.988	0.9947
96	1.83	1.83	2.435	1.027	0.9938

r^2 is the regression in binding equilibrium

Table 30. Binding parameters obtained from the fluorescence quenching method

Analysis of quenching mechanism was confirmed from the values of biomolecular quenching rate constants, which are evaluated using the equation (2):

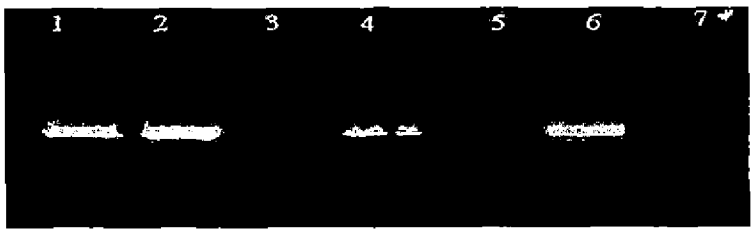
$$K_q = \frac{K_{SV}}{\tau_o} \qquad (2)$$

where τ_o is the lifetime of the biomolecules without the quencher and K_q stands for the bimolecular quenching constant. The value of τ_o for biopolymers is 10^{-8}s.[78] Accordingly the values of K_q are calculated **(Table 30)**. In general, the maximum K_q of various kinds of biomolecules is 10^{10} $M^{-1}s^{-1}$, which is less than the values in our case, indicates the fluorescence quenching is initiated by a static quenching process.[78] When small molecules bind independently to a set of equivalent sites on a biomacromolecule by static quenching, the equilibrium between free and bound molecules is given by the equation (3):

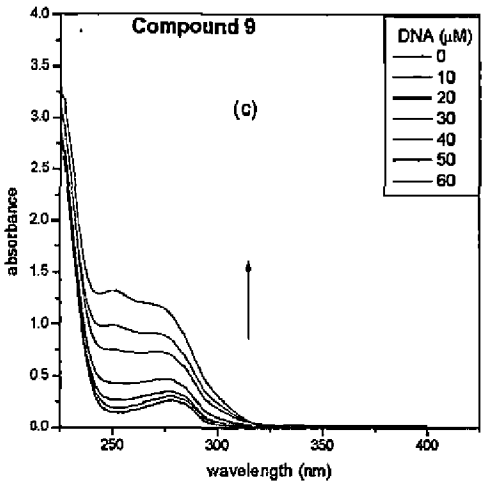
$$\log\frac{F_o - F}{F} = \log K + n\log[Q] \qquad (3)$$

where K and n are the binding constant and the number of binding sites, respectively. From a plot of $(F_o - F)/F$ vs. log $[Q]$ (**Fig. 21A**), K and the n can be obtained from the intercept and the slope (**Table 30**). As seen in **Table 30**, the values of n were approximately equal to 1, which indicated the existence of just one main binding site on DNA for compound **94-96**. Recently Ruiz et al.[79] have reported the incorporation of steroidal moiety in Rhodium and Iridium complexes, enhances their antitumor activities. These metal-steroidal complexes also have binding constants in a range of 10^3 M^{-1}. Haramane (HM), photosensitizer interact with DNA through intercalative mechanism and the binding constant (9×10^3 M^{-1}) is found to be on the same order of magnitude as that are in present case.[80]

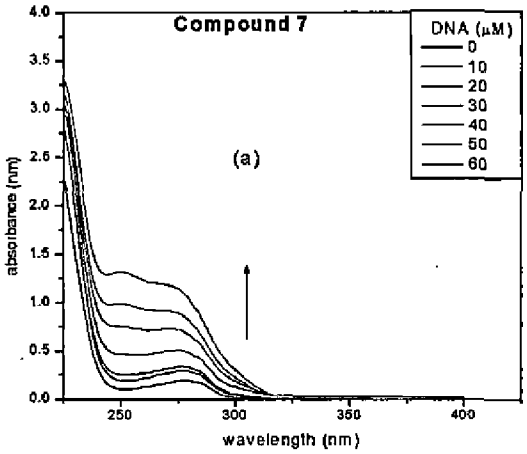
Molecular Docking: In our experiment, rigid molecular docking (two interacting molecules were treated as rigid bodies) studies were performed to predict the binding modes of compounds with a DNA duplex of sequence d(CGCAAATTTCGC)$_2$ dodecamer (PDB ID:1BNA) and provide an energetically favorable docked structures (representative figure of DNA-compound **95** complex shown in **Fig. 23**). It is evident from the figure that these type of compounds get attached with DNA through major groove and their pyrimidine moiety showed intercalation between the nucleotide base pairs of DNA. In this configuration, the group at 3β-axial position (i.e., X–moiety) remains inclined towards the phosphodiester bond of DNA and the possibility of hydrogen bonding cannot be ruled out. The resulting relative binding energy of docked steroidal pyrimidine–DNA complexes was found to be −280 to −293 kJ mol^{-1}. This value is consistent with the high binding constant obtained from spectroscopic values. Thus there is a mutual complement between spectroscopic techniques and molecular modeling, which can provide valuable information about the interaction of the compounds with DNA and the conformation constraints for adduct formation.



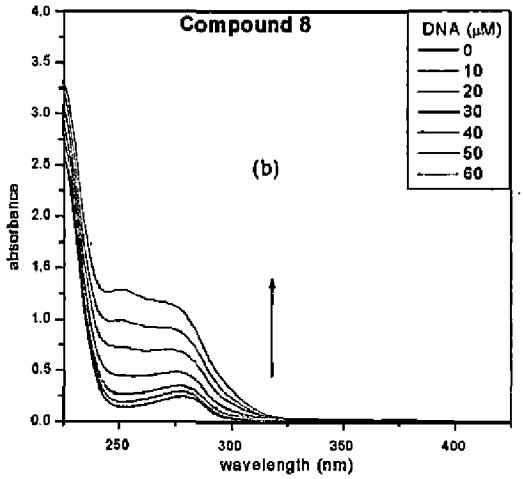
g. 20. Agrose gel electrophoresis of reaction mixtures containing pUC19 DNA and pound **94-96**. The concentration of DNA was 75 mg/L. Lane 1, DNA; lane 2, 3 DNA + 100, 200 μM); lane 4, 5 DNA + **95** (100, 200 μM); lane 6, 7 DNA + **96** (100, 200 μM)



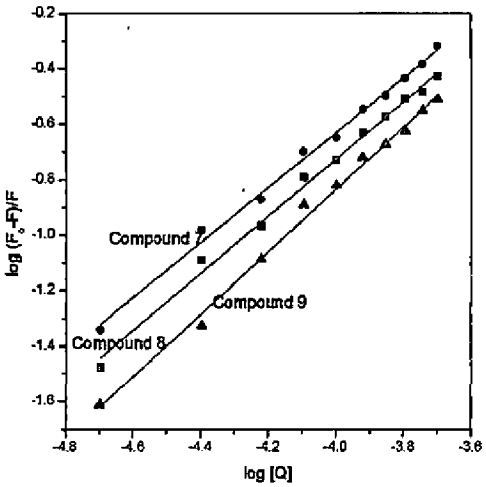
Compound **94**



Compound **95**

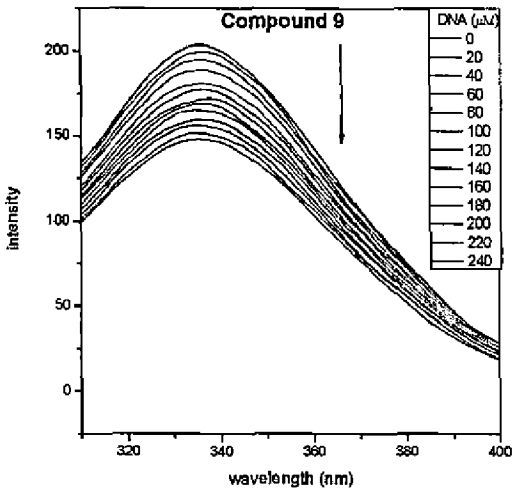


Compound **96**

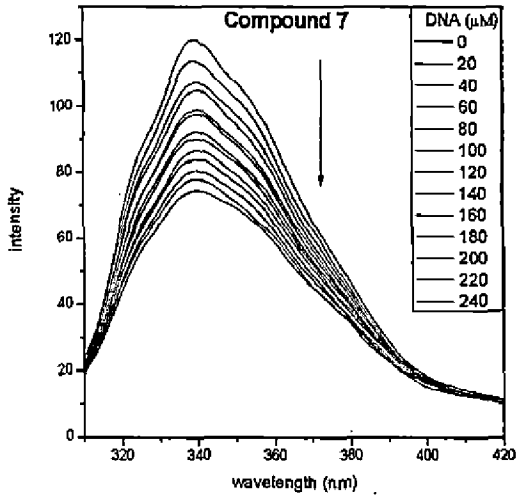


(A)

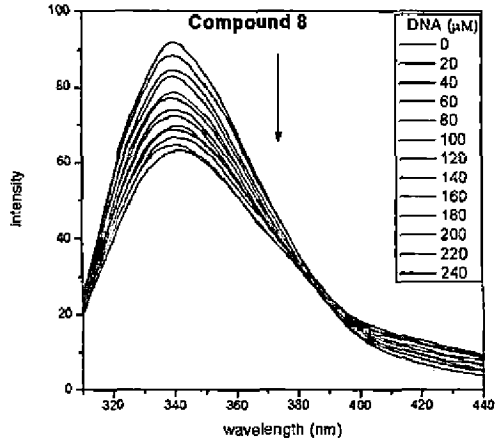
Fig. 21. Variation of UV-vis absorption for steroidal pyrimidines (**94-96**) with increase in concentration of CT DNA, **(A)** shows the variation of $(F_o - F)/F$ with log concentratio DNA.



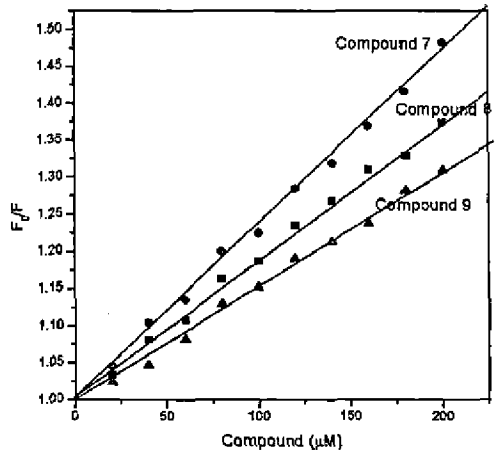
(a) Compound **94**



(b) Compound **95**

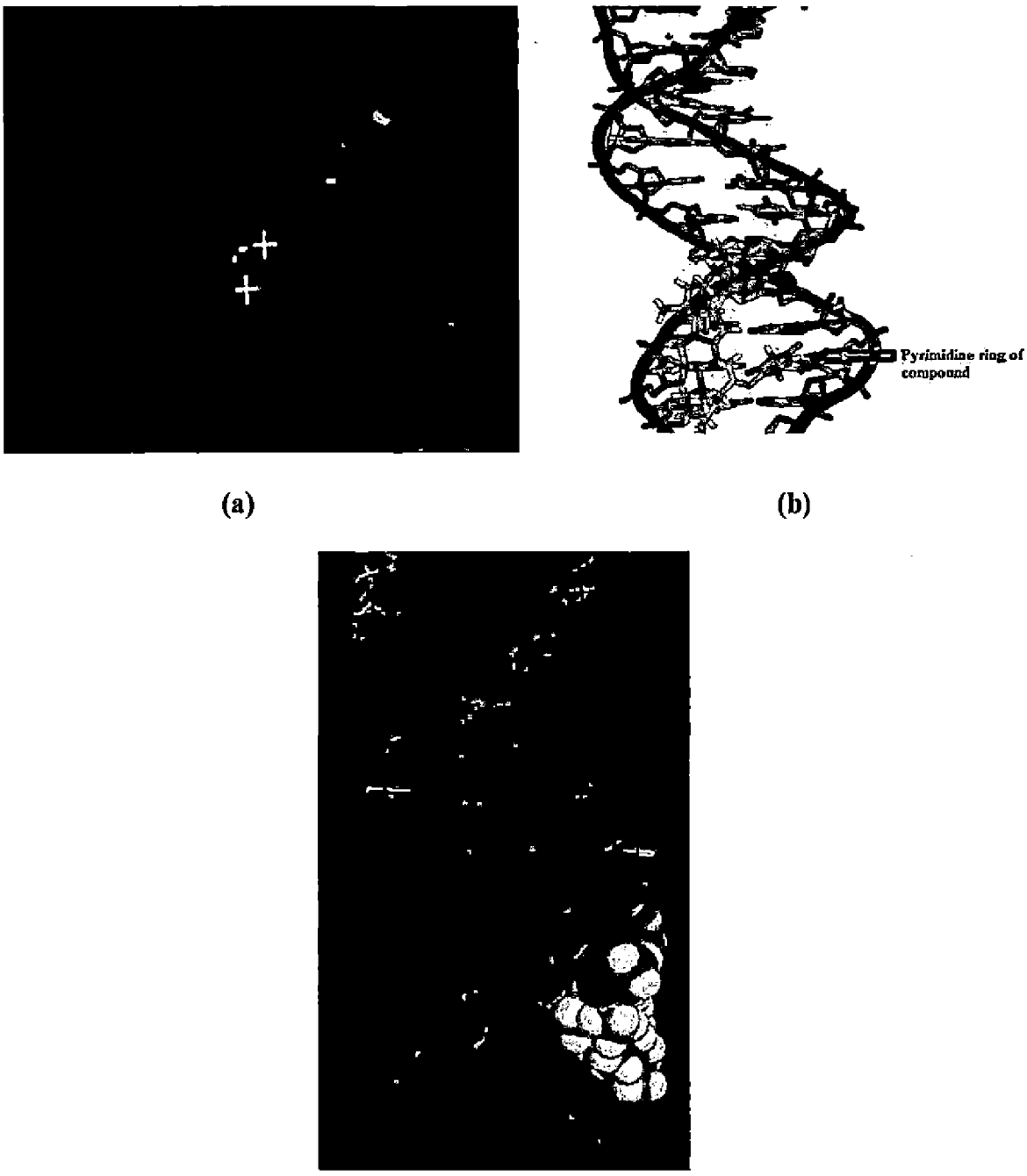


(c) Compound **96**



(d)

Fig. 22. Fluorescence titration of compound **94-96** with the CT DNA. Fluorescence intensity decreases with subsequent addition of DNA solution. (a), (b) and (c) represent fluorescence quenching of compound **94-96** due to the addition of DNA, respectively. (d) Stern-Volmer plots for the compound **94-96**.



(a) (b)

(c)

Fig. 23. Cartoon representation of DNA-compound **95**. The N-, S- and the O- termini of the pyrimidine moiety of the compound are shown as blue, yellow and red sticks, respectively. (a), (b) and (c) shows minimum energy poses of DNA-steroidal pyrmidine complex.

Work published;

Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Molecular Structure* 1045 (2013) 62-71

DNA binding studies of [6'-amino-2'-thioxo-4'-oxo dihydro-1H-pyrimidin-1-yl] 6-imino-5α-cholestane derivatives (97-99)

Electronic absorption titration: Electronic absorption spectroscopy is one of the most useful techniques for the investigation of mode of the interaction of compounds with DNA. The UV-vis spectra of compound **97-99** (refer to **chapter 1** for synthesis) exhibited intense absorption bands at 290 nm attributed to the $\pi \rightarrow \pi^*$ or intraligand transitions as shown in **Fig. 24**. This intense ligand ($\pi \rightarrow \pi^*$) absorption band is used to monitor the interaction of the compounds with CT DNA. Upon the addition of increasing concentration of CT DNA (0.70 - 4.24×10^{-5} M) to the test compounds in 2% DMSO/5mM Tris HCl/50mM NaCl buffer solution, the ligand intraligand absorption band exhibited hyperchromism without shift in band position. Hyperchromicity and hypochromicity are the spectral features of DNA concerning its double helical structure. The hyperchromic effect reflects the corresponding changes of DNA in its conformation and structure and results from the structural damage to the secondary structure DNA double helix.[81] These spectral characteristics suggested that compounds have higher binding propensity with CT DNA and interacts presumably by electrostatic interaction *via* the phosphate backbone of DNA double helix together with the hydrophobic interaction, since the bulky nature of the compounds is expected to hinder the intercalative mode of binding. The hydrophobic interaction with DNA replaces the water molecules in the DNA grooves leading to the enhancement in entropy and stabilization of the DNA-bound compound.[82]

In order to further compare the binding strength of the compounds, their intrinsic binding constant (K_b) were determined from the following equation (4).[83]

$$[DNA]/|\varepsilon_a - \varepsilon_f| = [DNA]/|\varepsilon_b - \varepsilon_f| + 1/K_b|\varepsilon_b - \varepsilon_f| \quad (4)$$

where, [DNA] represents the concentration of CT DNA, ε_a, ε_f and ε_b are the apparent extinction coefficients $A_{obs}/[M]$, the extinction coefficient for free compound and the extinction coefficient for compound in the fully bound form, respectively. In the plots of [DNA] / ε_a-ε_f versus [DNA], K_b is given by the ratio of the slope to the intercept.

The intrinsic binding constants for compound **97-99** were found to be 9.34×10^3, 6.56×10^3 and 1.54×10^4, respectively. The results obtained revealed that compound **99** binds more strongly with CT DNA as compared to the remaining compounds and the order of binding affinity is **99>97>98**.

Fluorescence spectroscopy: The emission spectra of compound **97-99** displayed intense luminescence at 358 nm at room temperature in the absence of DNA, when excited at 290 nm. On addition of increasing concentration of CT DNA (0.70×10^{-5} to 4.24×10^{-5} M) to the fixed amount of compounds (1×10^{-4} M), the emission intensity appreciably increases as shown in **Fig. 25**. The enhancement in the emission intensity is largely due to the change in environment around compounds and related to the extent to which the molecule is inserted into the hydrophobic environment of DNA minor and major grooves. Since DNA is a hydrophobic molecule, it reduces the accessibility of solvent molecules to reach the hydrophobic environment inside the DNA and the mobility of the compound is restricted at the binding site ultimately leading to decrease in vibrational mode of relaxation.[82] Furthermore, the binding of complex to the DNA helix could decrease the collisional frequency of solvent molecules with the compound, leading to the emission enhancement of the compound. These results revealed that interaction between CT DNA and compound occurs due to the hydrophobicity of both molecules.

To compare the binding affinity of compounds to DNA quantitatively, the binding constant 'K' and binding site number 'n' were calculated by using Scatchard equation (5) and (6).[84, 85]

$$C_F = C_T\,(F/F_o - P)\,(1 - P) \qquad (5)$$

$$r/c = K\,(n - r) \qquad (6)$$

where, C_F is the concentration of free compound, C_T is the total concentration of compound; F and F_o are fluorescence intensities in the presence and absence of DNA, respectively. P is the ratio of observed fluorescence quantum yield of the bound compound to that of the free compound. The value P was obtained as the intercept by extrapolating from a plot of F/F_o versus 1/[DNA], r denotes $C_B = (C_T - C_F) / [DNA]$, '**c**' is the free compound concentration and 'n' is the binding site number.

The binding constants for compound **97-99** were calculated to be 4.6×10^3, 3.5×10^3 and 1.1×10^4, respectively. The number of binding sites 'n' for compound **97-99** were found to be 1.34, 1.28 and 1.46, respectively indicating that compound **99** has higher DNA binding propensity in agreement with the electronic absorption titration experiment.

Nuclease or cleavage activity: The DNA cleavage was controlled by the relaxation of supercoiled circular form of pBR322 DNA into the nicked and linear form. When a circular plasmid DNA is subjected to agarose gel electrophoresis, the fastest migration will be observed for supercoiled form (Form I). If one strand is cleaved, the supercoils will relax to produce a slower moving open circular form (Form II). If both strands are cleaved, a linear form (Form III) will be generated that migrates in between Form I and Form II. The DNA cleaving ability of compound **99** was investigated using pBR322 DNA. In the absence of an activator, the compound **99** cleaved double stranded supercoiled plasmid DNA (SC form: Form I) (300 ng) in 5 mM Tris-HCl/50 mM NaCl buffer into nicked circular form (NC form: Form II) after 1 h of incubation at physiological pH 7.2 at 25 °C.[86, 87] Keeping the DNA concentration constant (300 ng) the concentration of compound **99** was varied (1.0-5.0 μM) and the cleavage reaction was monitored by gel electrophoresis. The concentration-dependent electrophoretic cleavage pattern exhibited conversion of SC form (Form I) to NC form (Form II) with increase in concentration of compound **99**.

At 3 μM concentration, compound **99** exhibited efficient nuclease activity. At still higher concentrations there was complete conversion of SC form into NC form with the concurrent formation of LC form. Presence of Form I, II and III of pBR322 DNA indicated that compound **99** is involved in cleavage of double strand super coiled DNA (**Fig. 26**).

The nuclease activity in the presence of activators *viz.*; MPA, GSH and H_2O_2 were also observed and the results showed significant enhancement in the cleavage activity. Their activating efficacy follows the order of GSH = H_2O_2 > MPA. To predict the mechanism of pBR322 plasmid DNA cleavage by compound **99**, comparative reactions were carried out in the presence of various radical inhibitors or trappers such as singlet oxygen scavenger sodium azide (NaN_3), hydroxyl radical scavengers *viz.*; dimethylsulfoxide (DMSO) *tert*-butyl alcohol (TBA) and superoxide dismutase (SOD) as superoxide anion inhibitor (**Fig. 27**). When the hydroxyl radical inhibitor DMSO and TBA were added to the reaction mixture, the nuclease activity was not inhibited, excluding the role of hydroxyl radical in the cleavage process. In the presence of radical scavengers like NaN_3 and SOD, the cleavage was inhibited under the present experimental conditions. The compound **99** seems to follow the mechanistic pathway involving singlet oxygen and superoxide anion to generate ROS responsible for initiating DNA strand scission.[88]

Molecular Docking: To understand steroidal pyrimidine-DNA interaction, the molecular docking technique is an attractive tool to get insight of the mechanistic study, by placing a molecule into the binding site of the target specific region of the DNA. In our experiment, rigid molecular docking (two interacting molecules were treated as rigid bodies) studies were performed to predict the binding modes of compounds with a DNA duplex of sequence d(CGCAAATTTCGC)$_2$ dodecamer (PDB ID: 1BNA), and provide an energetically favorable docked structures (DNA-compound **97-99** complexes shown in **Fig. 28**). Compound **97-99** are making one hydrogen bond with binding energy -266.22 Kcal, one hydrogen bond with binding energy -275.27 Kcal and three hydrogen bonds with binding energy -266.90 Kcal, respectively with DNA. The docking score of the compound **97-99** was found to be -6.0, -5.9 and -6.2, respectively revealing the better docking of compound **99** with DNA among the three compounds. It is evident from the docked poses that these types of compounds get attached with DNA through electrostatic as well as hydrophobic interaction. The steroid moiety due to its stereochemical reasons remains far from the nucleotide base pairs and hence it is the pyrimidine moiety with groups like -NH, -CO and -NH_2 which show intercalation between the nucleotide base pairs of DNA through hydrogen bonding. Thus binding energy and docking score values are consistent with the high binding constant obtained from spectroscopic values.

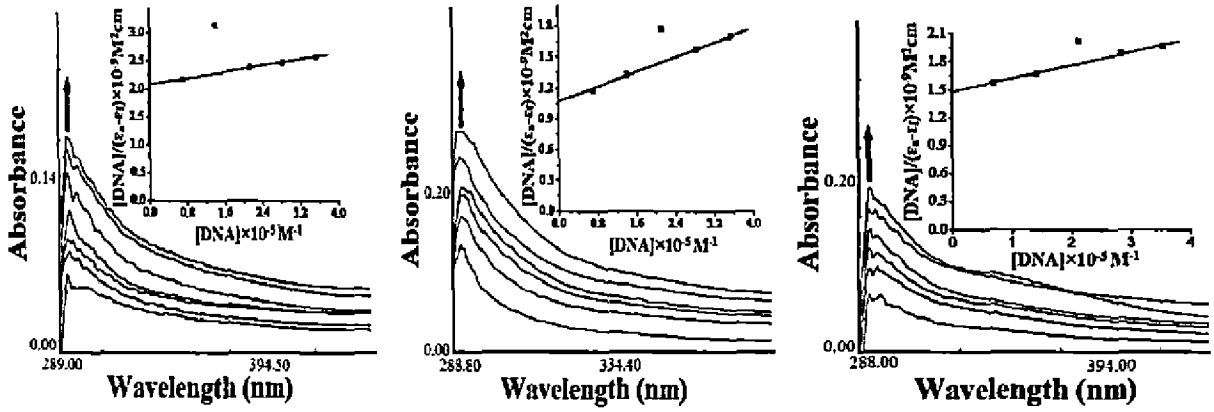


Fig. 24. Absorption spectra of compound **97-99** in Tris-HCl buffer upon the addition of CT DNA. [Compound] = 6.67×10^{-6} M, [DNA] = $(0.70–4.24) \times 10^{-5}$ M.

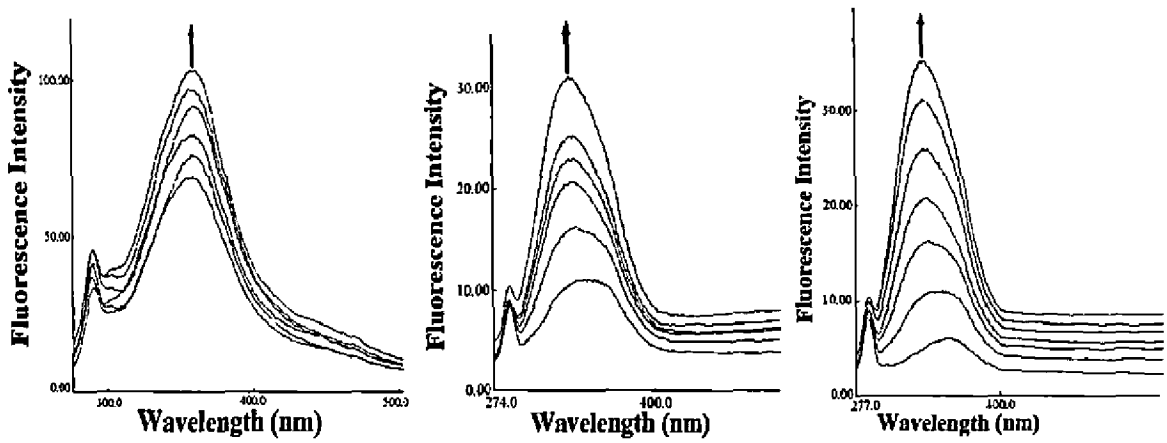
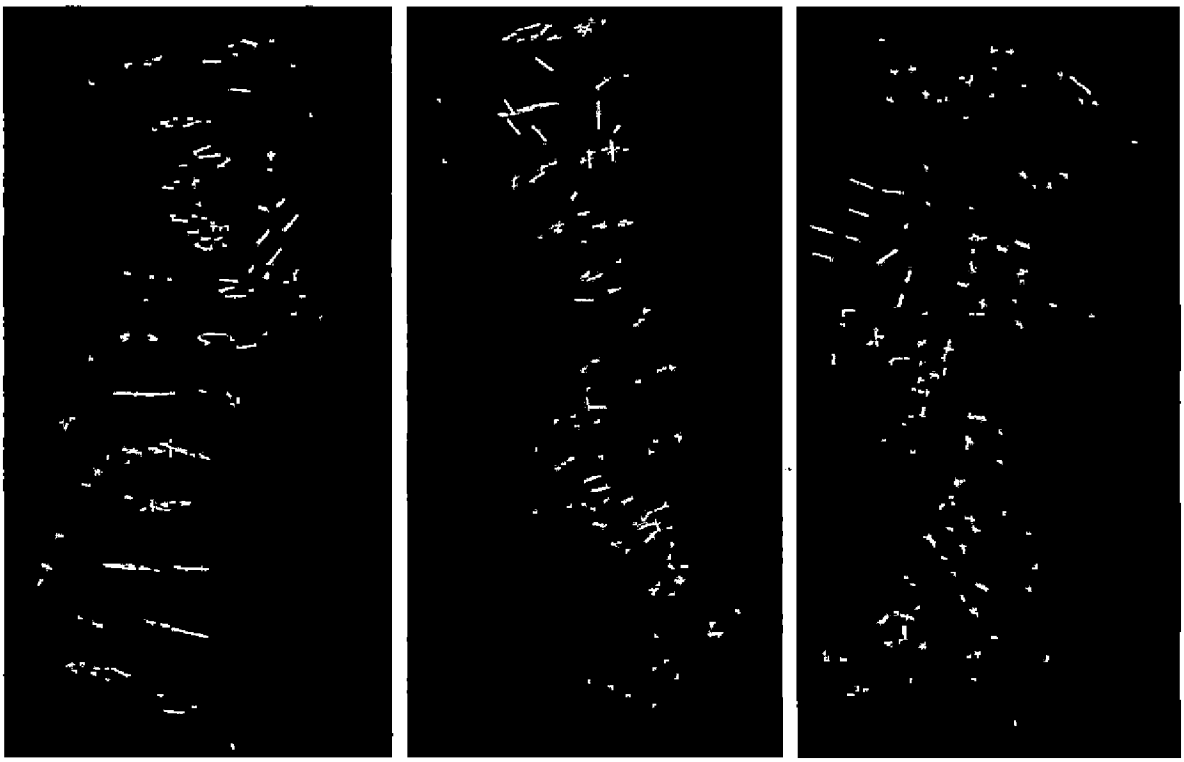


Fig. 25. The emission spectra of compound **97-99** in Tris-HCl buffer (pH 7.2) in the presence and absence of CT DNA at room temperature.

Fig. 26. Agarose gel electrophoresis patterns of pBR322 plasmid DNA (300 ng) cleaved by compound **99** (1.0-5.0 μM), after 1 h incubation time (concentration dependent) Lane 1: control; Lane 2: 1.0 μM **99** + DNA; Lane 3: 2.0 μM **99** + DNA; Lane 4: 3.0 μM **99** + DNA. Lane 5: 4.0 μM **99** + DNA; Lane 6: 5.0 μM **99** + DNA, in buffer (5 mM Tris-HCl/50 mM NaCl, pH 7.2 at 25 °C)

Fig. 27. Agarose gel electrophoresis pattern for the cleavage of pBR322 supercoiled DNA (300 ng) by compound **99** (2.0 μM) in presence of different activating agents and radical scavengers. Lane 1: **99** + GSH + DNA, Lane 2: **99** + MPA + DNA, Lane 3: **99** + H_2O_2 + DNA, Lane 4: **99** + NaN_3 + DNA, Lane 5: **99** + DMSO + DNA, Lane 6: **99** + t-butyl alcohol + DNA, Lane 7: **99** + SOD + DNA in buffer (5 mM Tris–HCl/50 mM NaCl, pH 7.2 at 25 °C)



(a) Compound **97** **(b)** Compound **98** **(c)** Compound **99**

Fig. 28. Docked poses of DNA with the compound **97-99**. The N, S and O termini of pyrimidine moiety of the compounds are shown as blue, yellow and red sticks, respectively.

Work published;

DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1H-pyrimidines, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Comptes Rendus Chimie*, http://dx.doi.org/10.1016/j.crci.2013.07.001 (in press)

DNA binding of 2'-amino-3'-carboethoxycholest-6-eno [5, 7- *d e*] 4H-pyran derivatives (63-65)

Gel Electrophoresis: The steroidal pyrans **63-65** (refer to **chapter 2** for synthesis) were examined for their binding abilities with pUC19 DNA by agarose gel electrophoresis. In the **Fig. 29**, lane 1 contains DNA only, lane 2, 4, 6 contains DNA and 100 μM of compound **63-65** and lane 3, 5, 7 contain DNA and 200 μM of compound **63-65**. At low concentration, compound **63-65** do not show any appreciable change in the band intensity of DNA (lane 2, 4, 6). However, at higher concentrations band intensity gets diminished in compound **63-65** (lane 3, 5, 7). Thus electrophoretic pattern demonstrate that the compound **63-65** interact with DNA. The loss in the band intensity at higher concentration may be assumed due to the intercalation of compound **63-65** to DNA, which in turn results in the displacement of EtBr. In case of lane 2, 4, 6 the concentration of compounds is not sufficient for displacement of EtBr. This was further supported from the fluorescence quenching, which gave convincing evidence for strong interaction of the compounds with DNA.

Electronic absorption titration: Absorption spectroscopy is one of the most useful techniques to study the mode of binding of any compound to DNA.[89] Addition of increasing amounts of CT DNA (0.70×10^{-5} - 4.24×10^{-5} M) to steroid pyrans **64** and **65** exhibited variation in the intense absorption bands at 210-340 nm as shown in **Fig. 30**. The absorption spectra of compound **64** and **65** reveal strong hyperchromism implying the higher DNA binding propensity of steroidal pyrans. Hyperchromicity and hypochromicity are the typical spectral features of DNA associated with its double helical structure. Hypochromic effect is attributed to the intercalative binding mode whereas hyperchromic effect is related to the electrostatic binding mode or to the partial uncoiling of DNA helix structure, exposing more bases of DNA indicative of strong binding of compounds to CT DNA.[90] Moreover, hyperchromic effect reflects the corresponding changes in the secondary structure of DNA in its conformation after the compound-DNA interaction.

To compare quantatively the effect of binding strength of compounds, the intrinsic binding constants K_b of the compound-DNA were determined by monitoring changes in the absorbance of π-π^* bands with increasing concentration of CT DNA. The intrinsic binding constant, K_b for compound **64** and **65** were calculated from Eq. (4), through a plot of [DNA]/ ε_a- ε_f vs. [DNA], where [DNA] represents the concentration of DNA and ε_a, ε_f and ε_b the apparent extinction coefficient (A_{obs}/[M]), the extinction coefficient for free compound (M)

and the extinction coefficient for the free steroid pyran (M) in the fully bound form, respectively and were found to be 5.3×10^3 and 3.7×10^3 M^{-1}, respectively.

$$[DNA]/|\varepsilon_a - \varepsilon_f| = [DNA]/|\varepsilon_b - \varepsilon_f| + 1/K_b|\varepsilon_b - \varepsilon_f| \quad (4)$$

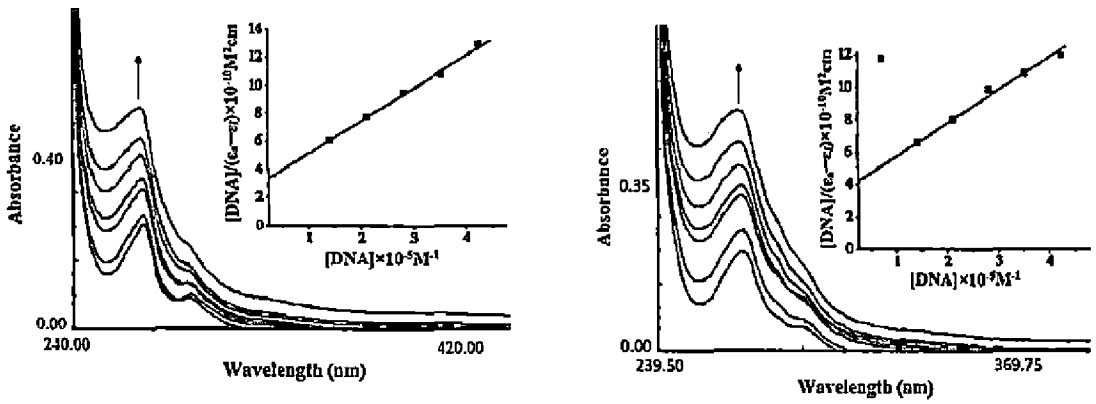
The reason for the higher binding affinity of compound **64** may be due to the presence of acetate group at the 3*β*-axial position of the steroid moiety which forms additional intercalative force with the base pairs of DNA molecule in addition to the hydrogen bonding by the $-NH_2$ of the pyran ring.

Fluorescence spectral studies: In the absence of CT-DNA, compound **64** and **65** emit luminescence in Tris-HCl buffer pH 7.2 at ambient temperature, with a fluorescence maximum appearing at 453 and 427 nm, respectively when excited at 270 nm. Upon addition of increasing concentration of [DNA] (0-33.3×10^{-6} M) to the fixed amount of compound concentration (6.67×10^{-6} M), there was a gradual enhancement in the fluorescence intensity of the compounds with no apparent change in the shape and position of the emission bands (**Fig. 31**). This implies that the compounds interacted with CT-DNA due to the inaccessibility of the solvent water molecules to reach the hydrophobic environment inside the DNA helix, and the mobility of the compound is restricted at the binding site ultimately leading to decrease in vibrational mode of relaxation. To compare the binding affinity of compound **64** and **65**, the binding data obtained from the emission spectra was fitted in the Scatchard equation to acquire the binding parameters.[91] A plot of r/Cf vs. r gave the binding constant for **64** and **65** as 2.85×10^3 and 1.17×10^3 M^{-1}, respectively. The number of steroid pyrans bound per DNA (n) calculated [92] for **64** and **65** was found to be 1.53 and 1.06, respectively; indicating that compound **64** interacted with DNA more strongly as compared to **65**.

Molecular docking: Molecular docking is a way to understand the mechanistic study by placing a molecule into the binding site of the target specific region of the DNA mainly in a non-covalent fashion,[93] which can substantiate the spectroscopic results. In our experiment, molecular docking studies of compound with DNA duplex of sequence $d(CGCGAATTCGCG)_2$ dodecamer (PDB ID: 1BNA) were performed in order to predict the chosen binding site along with preferred orientation of the molecules inside the DNA groove. The intercalation between the compounds and base pairs of DNA is mainly by hydrogen bonding shown by the NH_2 of the pyran ring. In the docked pose (**Fig. 32**), NH_2 of the compound forms two hydrogen bonds with the base pairs of DNA; one hydrogen bond is with the 2nd nitrogen of 4th guanine of DNA while another hydrogen bond is with the 3rd

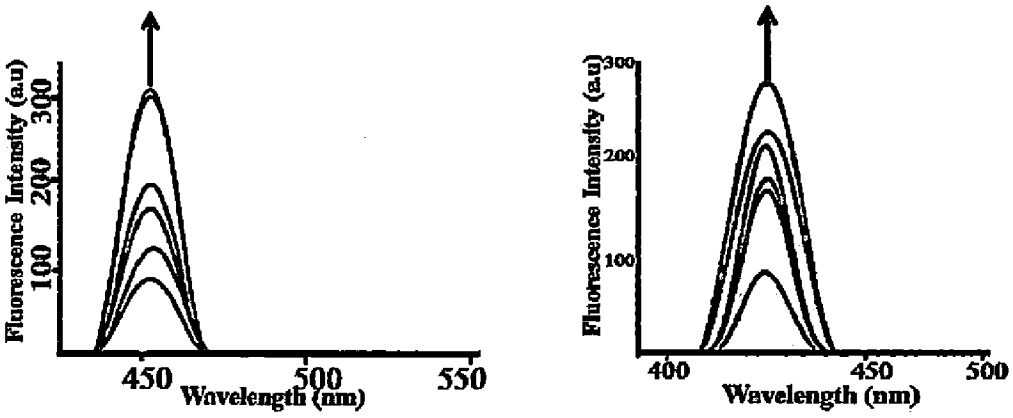
nitrogen of 5th guanine of DNA. The resulting binding energy of docked steroid pyran DNA complex was found to be -339.68 KJ mol^{-1}. Thus, we can conclude that there is a mutual complement between spectroscopic techniques and molecular docked model, which can be substantiate our spectroscopic results and at the same time provides further evidence of groove binding.

Fig. 29. Agarose gel electrophoresis of reaction mixtures containing pUC19 DNA and compound **63-65** Lane 1, DNA only; Lane 2, 3 DNA + compound **63** (100, 200 μM), Lane 4, 5 DNA + compound **64** (100, 200 μM) and Lane 6, 7 DNA + compound **65** (100, 200 μM)



Compound **64** Compound **65**

Fig. 30. Absorption spectra of compound **64** and **65** in Tris-HCl buffer upon the addition of calf thymus DNA [complex] = 6.67×10^{-6} M, [DNA] = $(0.70 - 4.24) \times 10^{-5}$ M. Arrow shows change in intensity with increasing concentration of DNA.



Compound **64** Compound **65**

Fig. 31. Emission spectra of compound **64** and **65** in the presence of DNA in 5 mM Tris-HCl/50 mM NaCl buffer. Arrows show the intensity changes upon increasing concentration of the DNA.

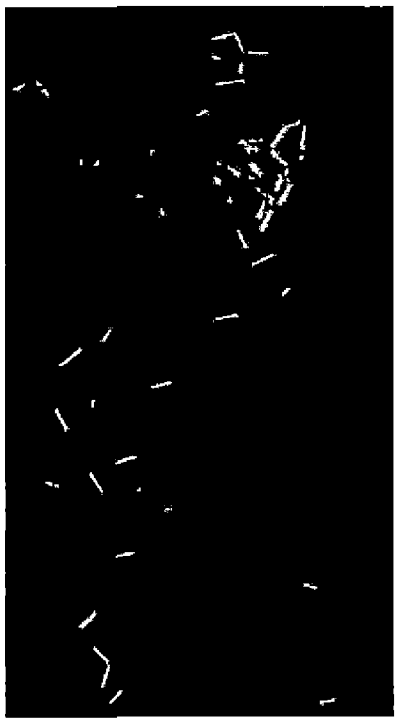


Fig. 32. Molecular docked model of steroidal pyran (yellow color) with DNA dodecamer duplex of sequence d(CGCGAATTCGCG)$_2$ (PDB ID: 1BNA) and the green dashed lines showing hydrogen bond interaction between them.

Work published;

Synthesis, molecular docking and biological evaluation of new steroidal 4H-pyrans, Shamsuzzaman, **Ayaz Mahmood Dar**, et al, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* 117 (2014) 493-501

DNA binding of 2'-amino-3'-cyanocholest-6-eno [5, 7- *d e*] 4H-pyran derivatives (66-68)

Electronic absorption titration: The covalent or non-covalent interactions are responsible for the binding of heterocyclic compounds with DNA. In covalent bonding, the easily leaving group of the compound is replaced by a nitrogen base of DNA such as guanine N7 while the non-covalent DNA interactions include electrostatic, intercalative and groove binding of heterocycles outside of a DNA helix. The absorption spectra of steroid pyrans **66-68** (refer to **chapter 2** for synthesis) exhibited hyperchromism of 26.11%, 27.51% and 19.63%, respectively at intraligand absorption band (267-271 nm) as shown in **Fig. 33.** The observed hyperchromism revealed that compound **66-68** bind to CT DNA electrostatically via non-covalent bonding with the DNA double helix. Moreover, steroid pyrans **66-68** exhibited a higher DNA binding profile due to the incorporation of NH_2 into the DNA binding grove. Since the hydrogen bonding interactions between -NH_2 groups of steroidal pyran and the functional groups positioned on the edge of DNA bases feature novelty as it provides molecular recognition at the specific site at the cellular target. The intrinsic binding constant values (K_b) of these compounds were determined by monitoring the changes in the absorbance at the intraligand band with increasing concentration of CT DNA. In order to further compare the binding strength of the compounds, their intrinsic binding constant (K_b) were determined from the following equation (4).

$$[DNA]/|\varepsilon_a - \varepsilon_f| = [DNA]/|\varepsilon_b - \varepsilon_f| + 1/K_b|\varepsilon_b - \varepsilon_f| \quad (4)$$

where, [DNA] represents the concentration of DNA, ε_a, ε_f and ε_b are the apparent extinction coefficients $A_{obs}/[M]$, the extinction coefficient for free compound and the extinction coefficient for compound in the fully bound form, respectively. In the plots of [DNA] / $\varepsilon_a - \varepsilon_f$ versus [DNA], K_b is given by the ratio of the slope to the intercept.

The binding constants K_b obtained for compound **66-68** are $1.97 \times 10^3\ M^{-1}$, $5.4 \times 10^3\ M^{-1}$ and $2.3 \times 10^3\ M^{-1}$, respectively. Interestingly, the intrinsic binding K_b value of compound **67** is higher in magnitude than other compounds. It may be due to the additional interaction like hydrogen bonding by the carbonyl moiety of acetate group ($OCOCH_3$) with DNA base pair which demonstrates the remarkably higher binding propensity of compound **67** towards CT DNA.

Fluorescence spectral studies: An intense luminescence at 336 nm in 0.01 Tris-HCl/50 mM NaCl buffer was obtained in the emission spectra of compound **66-68** at room temperature when excited at 269 nm. The emission intensity to a fixed amount of compounds gradually increases with no apparent change in the shape and position of the emission bands (shown in **Fig. 34**) on addition of increasing concentration of CT DNA (0.70×10^{-5} to 4.24×10^{-5} M). The enhancement in emission intensity is related to the extent to which the compound penetrates into the hydrophobic environment inside the DNA helix therefore compound mobility is restricted at the binding site leading to a decrease in the vibrational mode of relaxation and thus avoids the quenching effect of the solvent molecules. The increase in emission intensity revealed that the compound interacts by hydrophobic interaction in the DNA major groove.

To compare the binding affinity of compounds to DNA quantitatively, the binding constant 'K' and binding site number 'n' were calculated by using Scatchard equation (5) and (6).

$$C_F = C_T\,(F/F_o - P)\,(1 - P) \qquad (5)$$

$$r/c = K\,(n - r) \qquad (6)$$

Where, C_F is the concentration of free compound, C_T is the total concentration of compound; F and F_o are fluorescence intensities in the presence and absence of DNA, respectively. P is the ratio of observed fluorescence quantum yield of the bound compound to that of the free compound. The value P was obtained as the intercept by extrapolating from a plot of F/F_o versus 1/[DNA], r denotes $C_B = (C_T - C_F) / [DNA]$, '**c**' is the free compound concentration and 'n' is the binding site number.

The binding constant determined from the Scatchard equation for compound **66-68** was calculated to be $2.2 \times 10^3\ M^{-1}$, $5.37 \times 10^3\ M^{-1}$ and $2.51 \times 10^3\ M^{-1}$, respectively. The number of binding sites 'n' for compound **66-68** were found to be 0.92, 1.36 and 1.04, respectively indicating that compound **67** has higher DNA binding propensity in agreement with the electronic absorption titration experiment.

Chemical nuclease activity: Heterocyclic compounds have played an important role in DNA endonucleolytic cleavage reactions. DNA cleavage is normally reflected by relaxation of the supercoiled circular form (Form I) of pBR322 DNA resulting in nicked circular (Form II) and/or linear form (Form III). The DNA cleavage ability of the compound **66-68** was performed on pBR322 DNA (300 ng) incubated at 310 K with increasing concentration of compounds (5-25 μM) in aqueous buffer solution (5 mM Tris-HCl/50 mM NaCl, pH 7.2) for 1 h. As shown in **Fig. 35**, it was observed that all the three compounds are found to exhibit

nuclease activity at different concentration. The steroidal pyrans converted supercoiled DNA (Form I) into NC DNA (Form II) without concurrent formation of form III suggesting single strand DNA cleavage (Lane 2-16). Compound **67** showed efficient cleavage in comparison to **66** and **68**; with increase in concentration intensified nicked form (Form II) was observed.

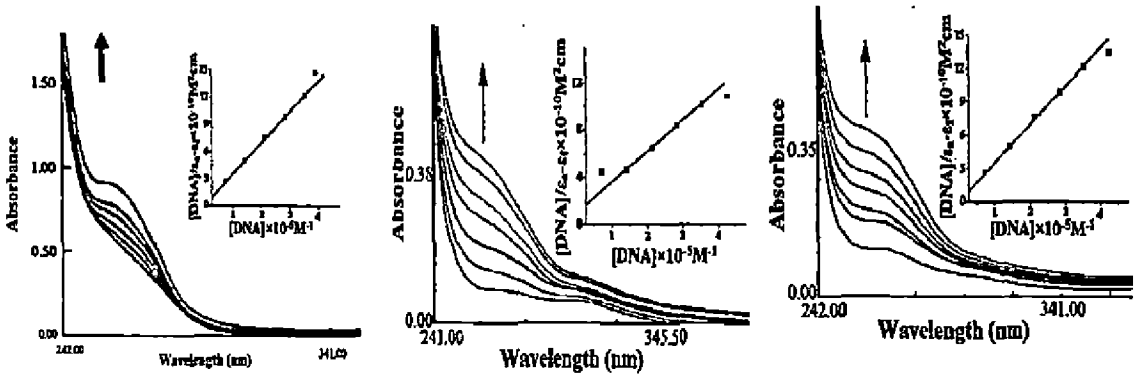
DNA cleavage in presence of recognition elements: The potential interacting site of compounds **66-68** with pBR322 DNA were determined in presence of minor groove binding agent (DAPI) and the major groove binding agent, methyl green (MG). The supercoiled pBR322 DNA was treated with DAPI or methyl green prior to the addition of compounds. The cleavage reaction mediated by compound **66-68** was inhibited in presence of DAPI while it remained unaffected in the presence of MG (**Fig. 36**) indicating minor groove-binding preference of the compounds.

DNA cleavage in presence of reactive oxygen species: In order to explore the mechanistic pathway of the cleavage activity, comparative DNA cleavage experiments of compound **66-68** were carried out in presence of some known radical scavengers such as DMSO and ethyl alcohol (EtOH) as hydroxyl radical scavenger ($HO^{\cdot}$), sodium azide (NaN_3) as singlet oxygen ($^1O_2$) quencher and superoxide dismutase (SOD) as superoxide anion radical ($O_2^{\cdot-}$) scavenger were used prior to the addition of compounds to DNA solution (**Fig. 37**). The addition of DMSO (Lane 2), EtOH (Lane 3) to compound **67** diminishes the cleavage activity which is indicative of the involvement of hydroxyl radical in the cleavage process. In the case of NaN_3 and SOD (Lane 4 and 5), the form II of plasmid DNA was converted to linear form III indicating two subsequent and proximate single strand breaks of DNA non-randomly. Similarly, compound **68** showed inhibition of DNA cleavage in presence of DMSO (Lane 6) whereas ethyl alcohol completely quench the formation of band II (Lane 7), suggestive of involvement of diffusible ($^{\cdot}$OH) hydroxyl radicals as one of the ROS responsible for DNA breakage. On the other hand, addition of NaN_3 and SOD did not show significant quenching of the cleavage revealing that singlet oxygen and superoxide anion were not involved in the cleavage process (Lane 8 and 9). Since, the compound **67** and **68** are able to cleave DNA in the absence of any reducing agent, which reveal that DNA might be cleaved by a discernible hydrolytic pathway. The compound **66** also showed inhibition of DNA cleavage in presence of DMSO (Lane 10) while as ethyl alcohol also quench the formation of band II (Lane 11), revealing the fact ($^{\cdot}$OH) hydroxyl radicals being responsible for DNA breakage. Since, the

compounds **66-68** are able to cleave DNA in the absence of any reducing agent, which reveals that DNA might be cleaved by a discernible hydrolytic pathway.

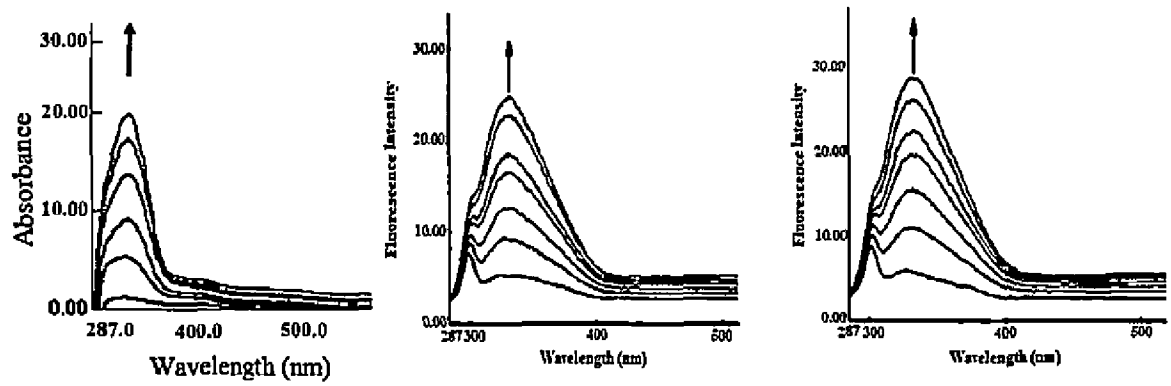
T4 ligation experiment: The standard DNA relegation protocol[94]was followed to support the hydrolytic mechanism of DNA cleavage and the DNA relegation experiments were performed using T4 ligase enzyme. The compound **66-68** treated with pBR322 plasmid DNA (2 mg), ligation buffer of 1.5 mL in 10X, T4 ligase 1 mL (2 units) and 2.5 mL of H_2O were mixed and incubated at 4 °C for 1 h. Subsequently, the samples were loaded on 1% agarose gel and visualized by staining with an ethidium bromide solution. To confirm the discernible hydrolytic DNA cleavage pathway mediated by compound **66-68,** DNA relegation experiment was performed in which supercoiled pBR322 DNA was treated with T4 ligase enzyme and subjected to gel electrophoresis.[95] Under present experimental conditions, the nicked form (Form II) was relegated to a large extent in the presence of T4 ligase enzyme in comparison to control DNA alone in supercoiled form (**Fig. 38**), providing a direct evidence in favour of hydrolytic mechanism.

Molecular Docking: In our experiment, molecular docking studies of compound **66-68** with DNA duplex of sequence $d(CGCGAATTCGCG)_2$ dodecamer (PDB ID: 1BNA) were performed in order to predict the chosen binding site along with preferred orientation of the molecules inside the DNA groove. The electrostatic interaction between the compounds and base pairs of DNA is mainly by hydrogen bonding shown by the NH_2 of the pyran ring. In **Fig. 39a**, the NH_2 of the compound **66** forms three hydrogen bonds with the base pairs of DNA; one hydrogen bond is with the 3nd oxygen of 15th cytosine of DNA; second with 5th oxygen of 16th guanidine of DNA while third hydrogen bond is with the 4th oxygen of 16th guanine of DNA. In the docked pose (**Fig. 39b**), NH_2 of the compound **67** forms two hydrogen bonds with the base pairs of DNA; one hydrogen bond is with the 2nd oxygen of 15th cytosine of DNA while second hydrogen bond is with the 3th oxygen of 16th guanine of DNA. In **Fig. 39c**, the oxygen of the pyran ring of compound **68** forms one hydrogen bond with the first oxygen of 13th cytosine of DNA. The resulting binding energy of docked [steroid 4H-pyrans (**66-68**)-DNA] complexes was found to be −308.61 KJ mol^{-1}, −311.34 KJ mol^{-1} and −301.42 KJ mol^{-1}, respectively. The more negative the relative binding, the more potent is the binding between DNA and target molecule. Thus, we can conclude that there is a mutual complement between spectroscopic techniques and molecular docked models, which can substantiate our spectroscopic results and at the same time provides further evidence of groove binding.



Compound 66 **Compound 67** **Compound 68**

Fig. 33. Absorption spectra of steroid pyrans **66-68** in Tris–HCl buffer upon the addition of CT DNA [complex] = 6.67×10^{-6} M, [DNA] = $(0.70–4.24) \times 10^{-5}$ M.



Compound 66 **Compound 67** **Compound 68**

Fig. 34. Emission spectra of compound **66-68** in Tris–HCl buffer (pH 7.2) in the presence and absence of CT DNA at room temperature

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 35. Gel electrophoresis diagram showing cleavage of pBR322 supercoiled DNA (300 ng) by compound **66-68** at 310 K after incubation for 1 h. Lane 1: DNA control; Lane 2: 5 μM of **67** + DNA; Lane 3: 10 μM of **67** + DNA; Lane 4: 15 μM of **67** + DNA; Lane 5: 20 μM of **67** + DNA; Lane 6: 25 μM of **67** + DNA; Lane 7: 5 μM of **68** + DNA; Lane 8: 10 μM of **68** + DNA; Lane 9: 15 μM of **68** + DNA; Lane 10: 20 μM of **68** + DNA; Lane 11: 25 μM of **68** + DNA. Lane 12: 5 μM of **66** + DNA; Lane 13: 10 μM of **66** + DNA; Lane 14: 15 μM of **66** + DNA; Lane 15: 20 μM of **66** + DNA; Lane 16: 25 μM of **66** + DNA.

Fig. 36. Gel electrophoresis pattern for the cleavage of pBR322 plasmid DNA (300 ng) by compound **66-68** in the presence of DNA minor and major groove binding agents; DAPI and methyl green at 310 K after incubation for 30 min. Lane 1: DNA; Lane 2: DNA+ **67** + DAPI (8 μM); Lane 3: DNA+ **67** + Methyl green (2.5 μL of a 0.01 mg ml^{-1} solution); Lane 4: DNA + **68** + DAPI (8μM); Lane 5: DNA + **68** + Methyl green (2.5 μL of a 0.01 mg ml^{-1} sol.); Lane 6: DNA + **66** + DAPI (8μM); Lane 7: DNA + **66** + Methyl green (2.5 μL of a 0.01 mg ml^{-1} sol.).

Fig. 37. Gel electrophoresis pattern for the cleavage pattern of pBR322 plasmid DNA (300 ng) by **66-68** (25 mM) in the presence of ROS at 310 K after incubation for 1 h. Lane 1, DNA control; Lane 2, DNA+ **67** + DMSO (0.4 μM); Lane 3, DNA+ **67** + ethyl alcohol (0.4 μM); Lane 4, DNA+ **67** + NaN_3 (0.4 μM); Lane 5, DNA+ **67** + SOD (15 Units); Lane 6, DNA + **68** + DMSO (0.4 μM); Lane 7, DNA + **68** + ethyl alcohol (0.4 μM); Lane 8, DNA + **68** + NaN_3 (0.4 μM); Lane 9, DNA+ **68** + SOD (15 Units). Lane 10, DNA + **66** + DMSO (0.4 μM); Lane 11, DNA + **66** + ethyl alcohol (0.4 μM); Lane 12, DNA + **66** + NaN_3 (0.4 μM); Lane 13, DNA + **66** + SOD (15 Units).

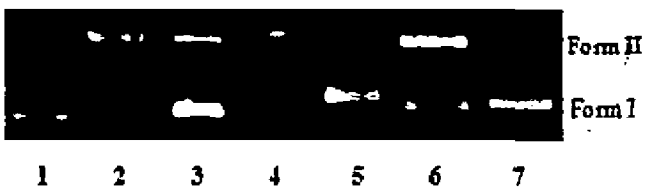
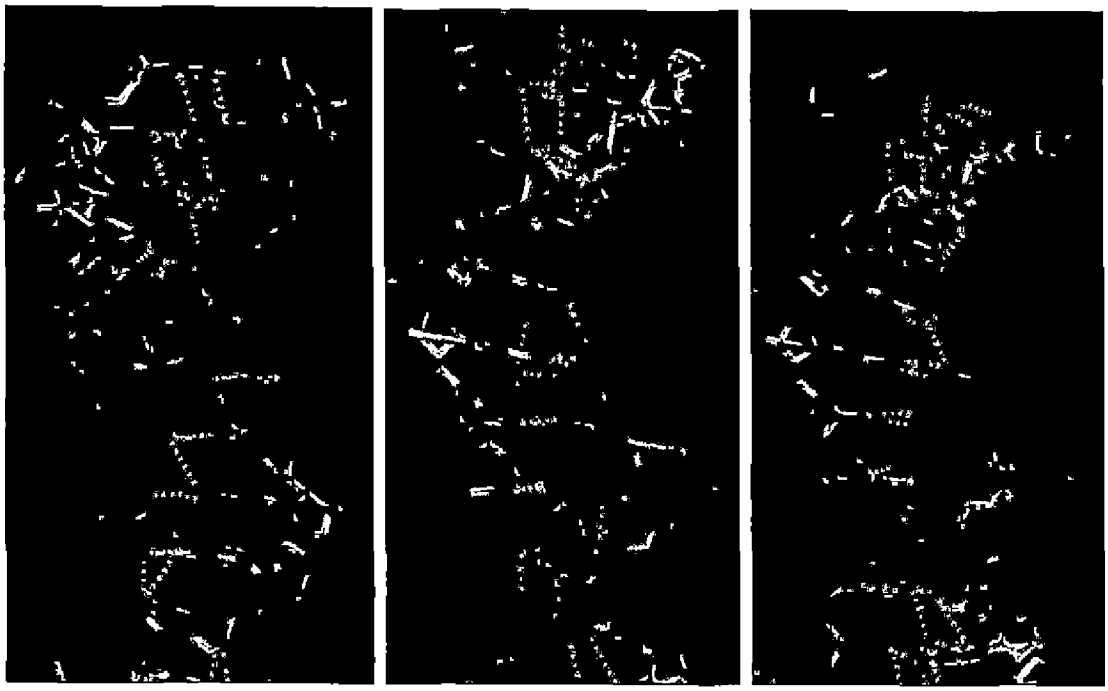
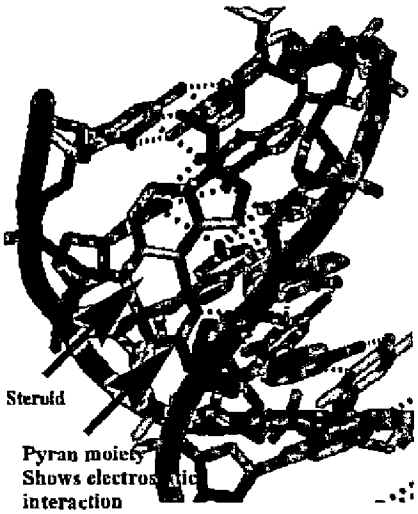


Fig. 38. Gel electrophoresis pattern for the pBR322 plasmid DNA ligation, linearized by compound **66-68**; Lane 1, DNA control; Lane 2, pBR322 plasmid DNA cleaved by **66**; Lane 3, ligation of nicked pBR322 plasmid DNA by T4 DNA ligase, Lane 4, pBR322 plasmid DNA cleaved by **67**, Lane 5, ligation of nicked pBR322 plasmid DNA by T4 DNA ligase. Lane 6, pBR322 plasmid DNA cleaved by **68**, Lane 7, ligation of nicked pBR322 DNA by T4 DNA ligase.



(66) (67) (68)



(A)

Fig. 39. Molecular docked models of steroidal pyrans **66-68** (purple colour) and docked pose (**A**) showing the general intercalation of compounds with DNA dodecamer duplex of sequence d(CGCGAATTCGCG)$_2$ (PDB ID: 1BNA) and the yellow dashed lines showing hydrogen bond interaction.

Work published;

Synthesis and biological studies of steroidal pyran based derivatives, Shamsuzzaman, **Ayaz Mahmood Dar**, et al., *Journal of Photochemistry and Photobiology B: Biology* 129 (2013) 36-47

References

References

1. A. H. Bandey, I. M. Zargar, B. A. Ganaie, *Steroids*, **76**, 1358 (2011)
2. P. B. Savage, *Curr. Med. Chem. Anti-Infect. Agents*, **3**, 293 (2002)
3. F. Qadri, A. M. Svennerholm, A. S. G. Faruque, R. B. Sack, *Clin. Microbiol. Rev.*, **18**, 465 (2005)
4. W. Zhang, E. M. Berberov, J. Freeling, D. He, R. A. Moxley, D. H. Francis, *Infect. Immun.*, **74**, 3107 (2006)
5. C. M. Nolan, G. E. Chalhub, G. D. Nash, T. Yamauchi, *Antimicrob. Agents Chem.*, **16**, 171 (1979)
6. A. S. Puerto, J. G. Fernandez, J. D. D. Castillo, M. J. Pino, G. P. Angulo, *Diagn. Micro. Infect. Dis.*, **54**, 135 (2006)
7. K. Visvanathan, N. E. Davidson, *Oncology*, **17**, 335 (2003)
8. X. Li, S. M. Singh, J. Cote, S. Laplante, R. Veilleux, F. Labrie, *J. Med. Chem.*, **38**, 1456 (1995)
9. Q. He, D. Jiang, *Leuk. Res.*, **23**, 369 (1999)
10. K. A. Latham, A. Zamora, H. Drought, S. Subramanian, A. Matejuk, H. Offner, E. F. Rosloniec, *J. Immunol.*, **17**, 5820 (2003)
11. D. Hosking, C. E. Chilvers, C. Christiansen, P. Ravn, R. Wasnich, P. Ross, M. McClung, A. Balske, D. Thompson, M. Daley, A. J. Yates, *N. Engl. J. Med.*, **338**, 485 (1998)
12. V. Gautam, M. Sharma, M. Panwar, N. Gautam, A. Kumar, I. K. Sharma, D. C. Gautam, *Phosphorus Sulfur, and Silicon*, **184**, 3090 (2009)
13. R. R. Gupta, M. Jain, R. S. Rathore, A. Gupta, *J. Fluorine Chem.*, **62**, 191 (1993)
14. Y. Deng, Y. Wang, C. Cherian, Z. Hou, S. A. Buck, L. H. Matherly, A. Gangjee, *J. Med. Chem.*, **51**, 5052 (2008)
15. L. F. Kuyper, J. M. Garvey, D. P. Baccanari, J. N. Champness, D. K. Stammers, C. R. Beddell, *Bioorg. Med. Chem.*, **4**, 593 (1996)
16. P. K. Andrus, T. J. Fleck, J. A. Oostveen, E. D. Hall, *J. Neurosci. Res.*, **47**, 650 (1997)
17. J. L. Wang, D. Liu, Z. J. Zhang, S. Shan, X. Han, S. M. Srinivasula, C. M. Croce, E. S. Alnemri, Z. Huang, *PNAS*, **97**, 7124 (2000)
18. I. J. S. Fairlamb, L. R. Marrison, J. M. Dickinson, F. J. Lu, J. P. Schmidt, *Bioorg. Med. Chem.*, **12**, 4285 (2004)
19. E. P. Das, S. J. Falcao, R. M. Melo, M. T. J. Srivastava, S. C. D. Catanho, Nascimento, *Eur. J. Med. Chem.*, **41**, 276 (2006)

20. B. C. Bookser, B. G. Ugarkar, M. C. Matelich, R. H. Lemus, M. Allan, M. Tsuchiya, M. Nakane, A. Nagahisa, J. B. Wiesner, M. D. Erion, *J. Med. Chem.*, **48**, 7808 (2005)
21. C. J. Valko, J. Rhodes, M. Moncol, M. Izakovic, M. Mazur, *Chem-Bio. Int.*, **160**, 1(2006)
22. W. Droge, *Physiol. Rev.*, **82**, 47 (2002)
23. D. V. Ratnam, D. D. Ankola, V. Bhardwaj, D. K. Sahana, M. N. V. R. Kumar, *J. Control. Release*, **113**, 189 (2006)
24. S. Suzen, *Top. Heterocycl. Chem.*, **11**, 145 (2007)
25. M. I. El-Zahari, S. S. Abdel-Karim, M. E. Haiba, M. A. Khedr, *Acta Poloniae Pharma. Drug Res.*, **68**, 357 (2011)
26. V. V. Dabholkar, R. P. Gavande, *Acta Poloniae Pharm. Drug Res.*, **69**, 247 (2012)
27. G. Naganagowda, P. Thamyongkit, R. Klai-U-dom, W. Ariyakriangkrai, A. Luechai, A. Petsom, *J. Sulfur Chem.*, **32**, 235 (2011)
28. B. Parashar, S. Bhardwaj, S. Sharma, G. D. Gupta, V. K. Sharma P. B. Punjabi, *J. Chem. Pharm. Res.*, **2**, 33 (2010)
29. N. A. A. El-Latif, A. E. E. Amr, A. A. Ibrahiem, *Monatsh. Chem.*, **138**, 559 (2007)
30. X. F. D. Feng, Y. Qua, X. Zhang, J. Wang, P. M. Loiseau, G. Andrei, R. Snoeck E. D. Clercq, *Bioorg. Med. Chem. Lett.*, **20**, 809 (2010)
31. H. P. Devnath, M. R. Islam, *Bangladesh J. Pharmacol.*, **5**, 30 (2010)
32. G. Marriapan, B. P. Saha, L. Sutharson, A. Haider, *Indian J. Chem.*, **49**B, 1671 (2010)
33. V. S. R. Chunduru, V. R. Rao, *J. Sulfur Chem.*, **31**, 545 (2010)
34. M. Kumar, K. Sharma, R. M. Samarth, A. Kumar, *Eur. J. Med. Chem.*, **45**, 4467 (2010)
35. V. K. Tandon, H. K. Maurya, N. N. Mishra, P. K. Shukla, *Eur. J. Med. Chem.*, **44**, 3130 (2009)
36. V. K. Tandon, H. K. Maurya, A. Tripathi, G. B. Shivakeshava, P. K. Shukla, P. Srivastava, D. Panda, *Eur. J. Med. Chem.*, **44**, 1086 (2009)
37. Y. S. Sadanandam, M. M. Shetty, A. B. Rao, Y. Rambabu, *Eur. J. Med. Chem.*, **44**, 197 (2009)
38. M. Bakavoli, M. Nikpour, M. Rahimizadeh, M. R. Saberi, H. Sadeghian, *Bioorg. Med. Chem. Lett.*, **15**, 2120 (2007)
39. R. Cruickshank, J. P. Duguid, B. P. Marmion, R. H. A. Swain, *Medicinal Microbiology*, 12th Edn., vol. II, Churchill Livingstone, London, p. 196, 1975
40. A. H. Collins, *Microbiological Methods*, 2nd Edn., Butterworth, London. p. 235, 1976
41. Z. K. Khan, *In vitro and in vivo screening techniques for bioactivity screening and*

evaluation, Proc. Int. Workshop UNIDO-CDRI, p. 210, 1997

42. R. S. Varma, *Antifungal Agents: Past, Present and Future prospects*, National Academy of Chemistry & Biology, Lucknow, India. p. 55, 1998
43. T. F. Slater, B. Sawyer, U. Strauli, *Biochim. Biophys. Acta*, **77**, 383 (1963)
44. T. Mosmann, *J. Immunol. Meth.*, **65**, 55 (1983)
45. H. J. Woerdenbag, T. A. Moskal, N. Pras, T. M. Malingre, F. S. El-Feraly, H. H. Kampinga, A. W. T. Konings, *J. Nat. Prod.*, **56**, 849 (1993)
46. H. O. Saxena, U. Faridi, J. K. Kumar, S. Luqman, M. P. Darokar, K. Shanker, C. S. Chanotiya, M. M. Gupta, A. S. Negi, *Steroids*, **72**, 892 (2007)
47. C. V. Kavitha, M. Nambiar, C. S. A. Kumar, B. Choudhari, K. Muniyappa, K. S. Rangappa, S. C. Raghavan, *Biochem. Pharmacol.*, **77**, 348 (2009)
48. N. P. Singh, *Mut. Res.*, **455**, 111 (2000)
49. G. J. Quinlan, J. M. C. Gutteridge, *Biochem. Pharmacol.*, **36**, 3629 (1987)
50. F. A. Shamsi, S. Husain, S. M. Hadi, *J. Biochem. Toxicol.*, **11**, 67 (1996)
51. M. S. Ahmad, F. Fazal, A. Rahman, S. M. Hadi, J. H. Parish, *Carcinogenesis*, **13**, 605 (1992)
52. J. A. Badwey, M. L. Karnovsky, *Annu. Rev. Biochem.*, **49**, 695 (1980)
53. A. Gupte, R. J. Mumper, *Cancer Treat. Rev.*, **35**, 32 (2009)
54. J. F. S. Carvalho, M. M. C. Silva, J. N. Moreira, S. Simoes, M. L. S. Mela, *J. Med. Chem.*, **53**, 7632 (2010)
55. L. N. Putral, M. J. Bywater, W. Gu, N. A. Saunders, B. G. Gabrielli, G. R. Leggatt, A. J. Nigel, McMillan *Mol. Pharmacol.*, **68**, 1311 (2005)
56. C. Li, W. Qiu, Z. Yang, J. Luo, F. Yang, M. Liu, J. Xie, J. Tang, *Steroids*, **75**, 859 (2010)
57. C. Wang, L. Liu, H. Xu, Z. Zhang, X. Wang, H. Liu, *Steroids*, **76**, 491 (2011)
58. J. G. Cui, L. Fan, L. Huang, H. Liu, A. Zhou, *Steroids*, **74**, 62 (2009)
59. Y. J. Lee, E. Shacter, *J. Biol. Chem.*, **274**, 19792 (1999)
60. K. Elizabeth, M. N. A. Rao, *Int. J. Pharm.*, **58**, 237 (1990)
61. G. K. Jayaprakasha, L. J. Rao, K. K. Sakariah, *Bioorg. Med. Chem.*, **12**, 5141 (2004)
62. V. Jaishree, S. Badami, S. Bhojraj, *Planta Medica*, **75**, 12 (2009)
63. R. Prabhakaran, P. Kalaivani, R. Jayakumar, M. Zeller, A. D. Hunter, S. V. Renukadevi, E. Ramachandran, K. Natarajan, *Med. Chem. Res.*, **3**, 42, (2011)
64. S. Sreelatha, P. R. Padma, M. Umadevi, *Food Chem. Toxicol.*, **47**, 702 (2009)
65. M. Komiyama, J. Sumaoka, *Curr. Opin. Chem. Biol.*, **2**, 751 (1998)
66. J. Hong, Y. Jiao, J. Yan, W. He, Z. Guo, L. Zhu, J. Zhang, *Inorg. Chim. Acta*, **363**,

793 (2010)

67. M. R. Burger, *Chem. Rev.*, **98**, 1153 (1998)
68. F. H. Westheimer, *Science*, **235**, 1173 (1987)
69. Y. Jin, J. A. Cowan, *J. Am. Chem. Soc.*, **127**, 8408 (2005)
70. J. Smith, K. Ariga, E. V. Anslyn, *J. Am. Chem. Soc.*, **115**, 362 (1993)
71. U. Scheffer, A. Strick, V. Ludwig, S. Peter, E. Kalden, M. W. Gobel, *J. Am. Chem. Soc.*, **127**, 2211 (2005)
72. H. U. Quan, Z. K. Jun, J. H. Le, C. X. An, H. U. M. Lin, W. Shun, *Science China*, **55**, 1345 (2012)
73. A. K. Williams, S. C. Dasilva, A. Bhatta, B. Rawal, M. Liu, E. A. Korobkova, *Anal. Biochem.*, **422**, 66 (2012)
74. A. I. Dragan, J. R. Casas-Finet, E. S. Bishop, R. J. Strouse, M. A. Schenerman, C. D. Geddes, *Biophys. J.*, **99**, 3010 (2010)
75. D. Mustard, D. W. Ritchie, *Funct. Bioinf.*, **60**, 269 (2005)
76. W. L. Delano, *The PyMOL Molecular Graphics System, DeLano Scientific, San Carlos*, CA, USA, 2002.
77. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Plenum Press, New York, p. 454, 1983
78. D. M. Togashi, A. G. Ryder, *J. Florescence*, **18**, 519 (2008)
79. J. Ruiz, V. Rodriguez, N. Cutillas, K. G. Samper, M. Capdevila, O. Palacios, A. Espinosa, *Dalton Trans.*, **41**, 12847 (2012)
80. B. K. Paul, N. Guchhait, *J. Phys. Chem. B*, **115**, 11938 (2011)
81. O. Trott, A. J. Olson, *J. Comput. Chem.*, **31**, 455 (2010)
82. S. Tabassum, M. Zaki, F. Arjmand, I. Ahmad, *J. Photochem. Photobiol. B*, **114**, 108 (2012)
83. Z. Tabassum, M. Muddassir, O. Sulaiman, F. Arjmand, *J. Luminescence*, **132**, 2178 (2012)
84. S. Tabassum, G. C. Sharma, F. Arjmand, A. Azam, *Nanotechnology*, **21**, 195102 (2010)
85. S. Tabassum, G. C. Sharma, A. Asim, A. Azam, R. A. Khan, *J. Organomet. Chem.*, **713**, 123 (2012)
86. V. Rajendiran, M. Murali, E. Suresh, M. Palaniandavar, V. S. Periasamy, M. A. Akbarsha, *Dalton Trans.*, 2157 (2008)
87. S. Ramakrishnan, V. Rajendiran, M. Palaniandavar, V. S. Periasamy, B. S. Srinag, H. Krishnamurthy, M. A. Akbarsha, *Inorg. Chem.*, **48**, 1309 (2009)
88. D. S. Sigman, A. Mazumder, D. M. Perrin, *Chem. Rev.*, **93**, 2295 (1993)

89. B. D. Wang, Z. Y. Yang, P. Crewdson, D. Q. Wang, *J. Inorg. Biochem.*, **101**, 1492 (2007)
90. N. Shahabadi, S. Kashanian, M. Khosravi, M. Mahdavi, *Trans. Metal Chem.*, **35**, 699 (2010)
91. G. Scatchard, *Ann. New York Acad. Sci.*, **51**, 660 (1949)
92. D. Charbonneau, M. Beauregard, H. A. Tajmir-Riahi, *J. Phys. Chem. B*, **113**, 1777 (2009)
93. R. Rohs, I. Bloch, H. Sklenar, Z. Shakked, *Nucl. Acids Res.*, **33**, 7048 (2005)
94. X. Q. Chen, X. Peng, J. Wang, Y. Wang, S. Wu, L. Zhang, T. Wu, Y. Wu, *Eur. J. Inorg. Chem.*, **42**, 5400 (2007)
95. D. Mandal, M. Chauhan, F. Arjmand, G. Aromi, D. Ray, *Dalton Trans.*, 9183 (2009)

Journal of Molecular Structure 1045 (2013) 62–71

Contents lists available at SciVerse ScienceDirect

Journal of Molecular Structure

journal homepage: www.elsevier.com/locate/molstruc

Steroidal pyrimidines: Synthesis, characterization, molecular docking studies with DNA and *in vitro* cytotoxicity

Shamsuzzaman [a,*], Ayaz Mahmood Dar [a], Zahid Yaseen [a], Khursheed Alam [a], Altaf Hussain [b], Manzoor Ahmad Gatoo [c]

[a] Department of Chemistry, Aligarh Muslim University, Aligarh 202 002, India
[b] Indian Institute of Integrative Medicine, Jammu 180 001, India
[c] Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202 002, India

HIGHLIGHTS

- A synthesis of new series of steroidal pyrimidines has been performed.
- The interaction of compounds with DNA were evaluated by UV–vis and fluorescence spectroscopy.
- The interaction of compounds with DNA was also carried out with Gel electrophoresis and docking studies.
- The *K* values for compounds **7–9** indicate higher binding affinity of compounds towards DNA.
- The new compounds were tested for *in vitro* cytotoxicity against different cancer and non-cancer cells.

ARTICLE INFO

Article history:
Received 24 December 2012
Received in revised form 8 April 2013
Accepted 8 April 2013
Available online 23 April 2013

Keywords:
Pyrimidine
UV–vis
Fluorescence
Gel electrophoresis
Cytotoxicity

ABSTRACT

A series of new steroid pyrimidines (**7–9**) were synthesized by reacting steroidal thiosemicarbazones (**4–6**) with diethyl malonate. The new compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The interaction studies of compounds (**7–9**) with DNA were carried out by employing gel electrophoresis, UV–vis and fluorescence spectroscopy. The acting force between the compounds (**7–9**) and DNA was mainly hydrophobic while the other interactions like van der Waals, hydrogen bonding cannot be ruled out. The gel electrophoresis pattern also demonstrated that the compound **7** alone or in presence of Cu (II) causes the nicking of supercoiled pBR322 and it seems to follow the mechanistic pathway involving generation of hydroxyl radicals that are responsible for initiating DNA strand scission. The docking study of compounds (**7–9**) suggested that the intercalation of compounds in between the nucleotide base pairs might be due to the presence of pyrimidine moiety in steroid molecule. MTT assay was carried out to check the toxicity of new compounds (**7–9**) against the different human cancer as well as non-cancer cell lines A545, MCF-7, HeLa, HL-60, SW480, HepG2, HT-29, A549, 184B5, MCF10A, NL-20, HPC and HPLF. Apoptotic degradation of DNA in presence of steroidal pyrimidines (**7–9**) was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining (comet assay).

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Heterosteroids have been accredited with a great amount of attention over the years by medicinal chemists for drug discovery. The interesting structural and stereochemical features of the steroid nucleus provide additional fascination to the researchers, and thereby alterations in the steroidal skeleton have been envisaged to discover new chemical entities with a potential to afford some promising drugs of the future. The incorporation of a heterocyclic ring or a heteroatom in the steroid backbone affects the chemical properties of a steroid and often results in useful alterations in its biological activities [1]. Therefore, researchers are on a continuous pursuit to design and produce better heterosteroids, by following natural models.

From last few years, numerous molecules possessing pyrimidine moiety have been reported to exhibit a broad spectrum of biological activities such as anticancer [2,3], antiviral [4], antibacterial [5], antioxidant [6,7], anxiolytic [8] and antidepressant [9]. Furthermore, they possess anti-inflammatory [10] and analgesic activities which are well documented in the literature [11,12]. Besides this, pyrimidine derivatives have been explored for use as histamine and adenosine receptor antagonists as well as among several other biological receptors and modulators [13,14].

In the field of molecular biology and drug development, the cleaving agents of nucleic acid have attracted extensive attention

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).

0022-2860/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.molstruc.2013.04.033

C. R. Chimie xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Comptes Rendus Chimie

www.sciencedirect.com

Full paper/Mémoire

DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1*H*–pyrimidines

Shamsuzzaman [a,*], Ayaz Mahmood Dar [a], Sartaj Tabassum [a], Mehvash Zaki [a], Yusuf Khan [b], Aamir Sohail [c], Manzoor Ahmad Gatoo [d]

[a] *Department of Chemistry, Aligarh Muslim University, Aligarh, 202002 India*
[b] *International Centre for Genetic Engineering and Biotechnology, New Delhi, 110067 India*
[c] *Department of Biochemistry, Aligarh Muslim University, Aligarh, 202002 India*
[d] *Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, 202002 India*

ARTICLE INFO

Article history:
Received 20 March 2013
Accepted after revision 11 July 2013
Available online xxx

Keywords:
Pyrimidine
UV–vis
Fluorescence
Docking
PBR322
Cytotoxicity

ABSTRACT

A new series of steroidal pyrimidines (**7–9**) has been synthesized by reacting steroidal thiosemicarbazones (**4–6**) with ethyl cyanoacetate. The compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The interaction studies of compounds **7–9** with DNA were carried out by UV–vis and luminescence spectroscopy. Compounds (**7–9**) bind to DNA preferentially through electrostatic and hydrophobic interactions, with K_b values found to be $6.56 \times 10^3\,M^{-1}$, $1.54 \times 10^4\,M^{-1}$ and $9.34 \times 10^3\,M^{-1}$, respectively, indicating the higher binding affinity of compound **8** towards DNA. Gel electrophoresis pattern demonstrated that compound **8** shows strong interaction with DNA and that, during its cleavage activity with pBR322 DNA, it seems to follow the mechanistic pathway involving the generation of singlet oxygen and a superoxide anion, which are responsible for initiating DNA strand scission. The docking study suggested that the intercalation of compounds in between the nucleotide base pairs is due to the presence of a pyrimidine moiety in the steroid molecule. MTT assay was carried out to check the toxicity of new compounds **7–9** against the different human cancer as well as non-cancer cell lines A545, MCF-7, HeLa, HL-60, SW480, HepG2, HT-29, A549, 184B5, MCF10A, NL-20, HPC, and HPLF. Apoptotic degradation of DNA in the presence of steroidal pyrimidines **7–9** was analysed by agarose gel electrophoresis and visualized by ethidium bromide staining (comet assay).
© 2013 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

1. Introduction

Steroids have been the focus of important research throughout the scientific history. But the recent past has seen an exhaustive interest of research being diverted towards these biologically important molecules. It is probably because of the various advantages associated with steroid-based chemotherapeutics, as these compounds turn out to be non-toxic, less vulnerable to multidrug resistance and highly bioavailable because they are capable of penetrating the cell wall [1]. Although various modifications of steroids, including derivatization, cyclization, heterocyclization, etc., have been tried, as far as the literature precedents are concerned, little efforts have been made towards the efficient synthesis of steroidal based pyrimidines derivatives and simultaneous studies of *in vitro* DNA binding or of nuclease.

Pyrimidine moiety containing molecules have been shown to exhibit a broad spectrum of biological activities like anticancer [2,3], antiviral [4], antibacterial [5],

* Corresponding author.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).

1631-0748/$ – see front matter © 2013 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.crci.2013.07.001

Please cite this article in press as: , et al. DNA binding, docking studies, artificial nuclease activity and *in vitro* cytotoxicity of newly synthesized steroidal 1*H*–pyrimidines. C. R. Chimie (2013), http://dx.doi.org/10.1016/j.crci.2013.07.001

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 117 (2014) 493–501

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Synthesis, molecular docking and biological evaluation of new steroidal 4*H*-pyrans

Shams Uzzaman [a,*], Ayaz Mahmood Dar [a], Aamir Sohail [b], Sheraz Bhat [b], Mir Faisal mustafa [b] Yusuf Khan [c]

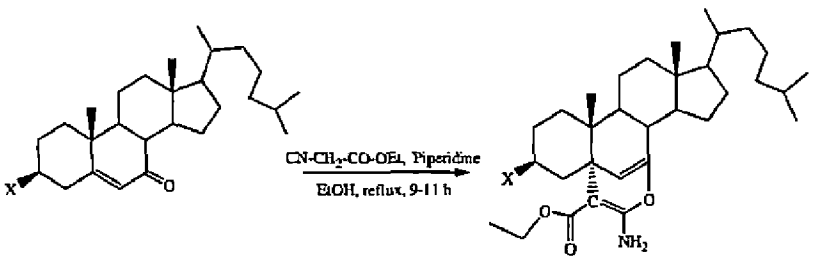
[a] Department of Chemistry, Aligarh Muslim University, Aligarh 202 002, India
[b] Department of Biochemistry, Aligarh Muslim University, Aligarh 202 002, India
[c] International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India

HIGHLIGHTS

- A convenient synthesis of new series of steroidal 4*H*-pyrans has been performed.
- The interaction of compounds **4** and **5** with DNA was evaluated by different biophysical techniques.
- The *K* values for compounds **4** and **5** indicate their higher binding affinity towards DNA.

GRAPHICAL ABSTRACT

A convenient synthesis of new series of steroidal 4*H*-pyrans has been performed. After characterization, the interaction of compounds **4** and **5** with DNA was evaluated by gel electrophoresis, docking studies and UV–vis and fluorescence spectroscopy. MTT assay and comet assay has been performed to check the *in vitro* cytotoxicity of new compounds.



X
OAc (1)
Cl (2)
H (3)

X
OAc (4)
Cl (5)
H (6)

ARTICLE INFO

Article history:
Received 25 May 2013
Received in revised form 27 July 2013
Accepted 3 August 2013
Available online 16 August 2013

Keywords:
4*H*-pyran
Ethyl cyanoacetate
Anticancer
Docking
Comet assay

ABSTRACT

A series of new steroidal 4*H*-pyrans (**4–6**) have been synthesized from steroidal α, β-unsaturated ketones (**1–3**). The products (**4–6**) were characterized by IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The interaction studies of compounds (**4–6**) with DNA were carried out by employing gel electrophoresis, UV–vis and fluorescence spectroscopy. The gel electrophoresis pattern revealed that compounds (**4–6**) bind to DNA and also demonstrated that the compound **6** alone or in presence of Cu (II) causes the nicking of supercoiled pBR322. The compounds **4** and **5** bind to DNA preferentially through electrostatic and hydrophobic interactions with K_b values found to be 5.3×10^3 and 3.7×10^3 M^{-1}, respectively, indicating the higher binding affinity of compound **4** towards DNA. The docking study suggested the intercalation of compounds in between the nucleotide base pairs. The cytotoxicity and genotoxicity of the newly synthesized compounds were checked by MTT and comet assay, respectively during which compound **6** showed potential behaviour.

© 2013 Elsevier B.V. All rights reserved.

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (S. Uzzaman).

1386-1425/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.saa.2013.08.019

Journal of Photochemistry and Photobiology B: Biology 129 (2013) 36–47

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Synthesis and biological studies of steroidal pyran based derivatives

Shamsuzzaman [a,*], Ayaz Mahmood Dar [a], Yusuf Khan [b], Aamir Sohail [c]

[a] Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India
[b] International Centre for Genetic Engineering and Biotechnology, New Delhi 110067, India
[c] Department of Biochemistry, Aligarh Muslim University, Aligarh 202002, India

ARTICLE INFO

Article history:
Received 6 July 2013
Received in revised form 17 September 2013
Accepted 25 September 2013
Available online 8 October 2013

Keywords:
4H-Pyran
DNA binding
Gel electrophoresis
MTT assay
Comet assay

ABSTRACT

Steroid based cancer chemotherapeutic agents of the type 2′-amino-3′-cyanocholest-6-eno[5,7-de]4H-pyrans (**1c–3c**) have been synthesized and characterized by the various spectroscopic and analytical techniques. The DNA binding studies of compounds (**1c–3c**) with CT DNA were carried out by UV–vis and fluorescence spectroscopy and gel electrophoresis. The compounds (**1c–3c**) bind to DNA preferentially through electrostatic and hydrophobic interactions with K_b values found to be 5.4×10^3, $2.3 \times 10^3\ M^{-1}$ and $1.97 \times 10^3\ M^{-1}$, respectively indicating the higher binding affinity of compound (**1c**) towards DNA. The molecular docking study suggested that the electrostatic interaction of compounds (**1c–3c**) in between the nucleotide base pairs is due to the presence of pyran moiety in steroid molecule. All the compounds (**1c–3c**) cleave supercoiled pBR322 DNA via hydrolytic pathway, as validated by T4 DNA ligase assay. The compounds (**1c–3c**) were screened for *in vitro* cytotoxicity against the cancer and non-cancer cells SW480, A549, HepG2, HeLa, MCF-7, HL-60, DU-145, NL-20, HPC and HPLF by MTT assay. The compounds (**1c–3c**) were tested for genotoxicity (comet assay) involving apoptotic degradation of DNA and was analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. The results revealed that compound (**1c**) has better prospectus to act as cancer chemotherapeutic candidate which warrants further *in vivo* anticancer investigations.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Steroids are a class of important polycyclic compounds which exhibit diverse biological activities. Except for the naturally occurring substances, most of steroidal pharmaceuticals are semi-synthetic compounds [1]. Several steroidal derivatives have been investigated as new curative agents for cancers and other diseases. It is proved that a number of biologically important properties of modified steroids are dependent upon structural features of the steroid ring system or side chain so this chemical modification of the steroid provides a way to alter the functional groups and numerous structure–activity relationships have been established by such synthetic alterations [2].

Pyran derivatives are of considerable interest in industry as well as in academia owing to their potential biological and medicinal activities, such as analgesic, anticancer, anti-inflammatory, antibacterial and also serve as potential inhibitors of human Chk-1 kinase (Fig. 1) [3]. Furthermore, the applications of pyran derivatives are not only found in pharmaceutical ingredients and biological agrochemicals [4] but they also constitute a structural unit of number of natural products [5].

DNA cleaving agents have attracted extensive attention in the field of molecular biology due to their potential applications [6]. Under uncatalyzed physiological conditions, the phosphodiester bonds of DNA are extremely stable and the half life of DNA hydrolysis is estimated to be around 200 million years [7]. Some of the metal complexes have been widely investigated as efficient cleaving agents of nucleic acids [8] but the serious issues over their lability and toxicity restricted the practical usage of these compounds in pharmacy [9]. To overcome these limitations, Gobel and co-workers [10] put forward the concept of 'metal free cleaving agents' which are being applied to active phosphodiesters like 'nucleic acid mimic' and RNA.

Although various modifications of steroids have been tried including derivatization, cyclization, and heterocyclization, very few efforts have been made towards the efficient synthesis and the study of DNA binding, cleavage, cytotoxic and genotoxic activity of steroid based 4H-pyrans. So in continuation of our previous work [11] herein, we report the synthesis of new steroidal 4H-pyrans as metal free DNA binding agents. The presence of —NH and —CO groups in the molecules can cooperatively participate in the interaction with DNA via hydrogen bonding. A computer aided molecular docking study was carried out to validate the specific binding mode of the compounds. Furthermore, these compounds

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).

1011-1344/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jphotobiol.2013.09.004

Available online at www.sciencedirect.com

ScienceDirect

Journal of Taibah University for Science xxx (2013) xxx–xxx

Journal of Taibah University for Science

www.elsevier.com/locate/jtusci

Structural, optical and antimicrobial studies of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones

Shamsuzzaman[a,*], Ayaz M. Dar[a], Suboohi Shervani[b], Irshad Bhat[c], Manzoor A. Gatoo[d]

[a] *Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India*
[b] *Centre of Excellence in Materials Science (Nanomaterials), Department of Applied Physics, Aligarh Muslim University, Aligarh 202002, India*
[c] *Department of Physics, Aligarh Muslim University, Aligarh 202002, India*
[d] *Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, India*

Received 8 April 2013; received in revised form 17 June 2013; accepted 21 August 2013

Abstract

A convenient synthesis of a new series of nano steroidal pyrazolones is reported. They were characterized by X-ray diffraction, scanning electron microscopy, UV–vis light, Fourier transform-infrared spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance, mass spectroscopy and analytical data. The X-ray diffraction patterns at room temperature showed that two nano steroids, **7** and **8**, are formed in single phase with hexagonal crystal symmetry, while nano steroid **9** is formed with orthorhombic crystal symmetry. Compounds **1–3** are formed with hexagonal, monoclinic and tetragonal crystal symmetry, respectively. Scanning electron microscopy showed that the crystals of nano steroids **7–9** are brick-shaped agglomerates with less sharp edges and a rough surface with a few microcrystals, while the homogeneous crystal of nano steroid **3** has an approximate spherical morphology of nano particles conjoined to the chains. UV–vis absorption analysis showed that the band gap energy of nano steroids **7** and **8** was 3.70 eV and 4.61 eV, while that of **2** and **3** was 4.38 eV and 4.27 eV, respectively. Nano steroids **2**, **3** and **7–9** were screened for antimicrobial activity against various strains; nano compound **7** showed potential antimicrobial activity.
© 2013 Taibah University. Production and hosting by Elsevier B.V. All rights reserved.

Keywords: Pyrazolone; Cyanoacetohydrazide; SEM; UV–vis; Particle size; Antimicrobial

1. Introduction

Self-assembly of organic compounds with cholesteryl groups has proved to be an attractive field in nanotechnology research. Some steroid derivatives are known to form ordered structures, which indicate thermotropic and lyotropic liquid crystalline, monolayers, multilayers and micelles [1]. As steroids are important constituents of most eukaroytic cell membranes, a great deal is known about certain aspects of their function. In model systems, cholesterol is distributed evenly on both sides of the bilayer, with its polar hydroxyl group held in the vicinity of the phosphate groups of phospholipids [2,3].

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).
Peer review under responsibility of Taibah University

Production and hosting by Elsevier

1658-3655 © 2013 Taibah University. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jtusci.2013.08.003

Please cite this article in press as: Shamsuzzaman, et al. Structural, optical and antimicrobial studies of 3β-acetoxycholest-5-ene, 3β-acetoxy-6-nitrocholest-5-ene and newly synthesized steroidal pyrazolones, J. Taibah Univ. Sci. (2013), http://dx.doi.org/10.1016/j.jtusci.2013.08.003

Arabian Journal of Chemistry (2013) xxx, xxx–xxx

King Saud University

Arabian Journal of Chemistry

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Anticancer and antimicrobial evaluation of newly synthesized steroidal 5,6 fused benzothiazines

Shamsuzzaman [a,*], Ayaz Mahmood Dar [a], Hena Khanam [a], Manzoor Ahmad Gatoo [b]

[a] *Department of Chemistry, Aligarh Muslim University, Aligarh 202 002, India*
[b] *Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202 002, India*

Received 11 May 2012; accepted 23 June 2013

KEYWORDS

Benzothiazine;
5α-Cholestan-6-one;
Antibacterial;
Antifungal;
Anticancer

Abstract A series of new 5α-cholestano [5,6-b] benzothiazines (**4–6**) has been synthesized by the reaction of 5α-cholestan-6-one (**1–3**) with 2-aminothiophenol in the presence of iodine. The structures of newly synthesized compounds have been established on the basis of spectral and analytical data. Compounds (**1–6**) were screened for in vitro anticancer activity against the human cancer cell lines; SW480 (colon adenocarcinoma cells), A549 (lung carcinoma cells), HepG2 (hepatic carcinoma cells) and HeLa (cervical cancer cells) using MTT assay during which the products (**4–6**) showed marked increase in anticancer activity and in particular, compound **6** showed IC_{50} = 13.73 μmol L^{-1} against HeLa cells, being more effective than Doxorubicin against the same cells. Compounds **4** and **6** also showed minimum IC_{50} of 15.83 μmol L^{-1} and 16.89 μmol L^{-1} against HepG2 and A549 cells, respectively. Compounds (**1–6**) were also tested for in vitro antimicrobial activity against different bacterial as well as fungal strains during which newly synthesized compounds (**4–6**) were found more potent than starting compounds (**1–3**). Compound **4** was found to be more potent than the reference drug, Chloramphenicol, in the case of *Escherichia coli* while compound **5** was found almost equally potential antifungal agent against P. marneffei in comparison with the reference drug, Nystatin.

© 2013 Production and hosting by Elsevier B.V. on behalf of King Saud University.

1. Introduction

Steroids have been the important focus of research throughout the scientific history. But the recent past has seen an exhaustive focus of research being diverted towards these biologically important molecules. This is pertinently true of the rational semi-synthetic modifications of steroidal molecules. Probably, it is because of the various advantages associated with steroid based chemotherapeutics. These compounds turn out to be non-toxic, less vulnerable to multi-drug resistance (MDR)

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).

Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

1878-5352 © 2013 Production and hosting by Elsevier B.V. on behalf of King Saud University.
http://dx.doi.org/10.1016/j.arabjc.2013.06.027

Please cite this article in press as: Shamsuzzaman, et al., Anticancer and antimicrobial evaluation of newly synthesized steroidal 5,6 fused benzothiazines. Arabian Journal of Chemistry (2013), http://dx.doi.org/10.1016/j.arabjc.2013.06.027

Med Chem Res
DOI 10.1007/s00044-013-0636-y

MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, evaluation and docking studies on steroidal pyrazolones as anticancer and antimicrobial agents

Shamsuzzaman · Ashraf Mashrai · Anis Ahmad · Ayaz Mahmood Dar · Hena Khanam · Mohd Danishuddin · Asad U. Khan

Received: 15 February 2013 / Accepted: 20 May 2013
© Springer Science+Business Media New York 2013

Abstract A series of new steroidal pyrazolones have been synthesized, characterised and evaluated for their in vitro anticancer activity. They were tested against five cancer (SW480, HepG2, A549, HeLa and HL-60) cell lines. The synthesized compounds showed high selectivity and compound **4** showed the strongest inhibitory activity against human SW480 (IC_{50} = 11.67 μmol L^{-1}). In addition, the synthesized compounds were tested for their antimicrobial activity by disc diffusion assay and MIC by broth micro dilution method against Gram-positive, Gram-negative strains of bacteria as well as fungus strains and we found a correlation between the observed and predicted antimicrobial activities. Docking studies were performed to investigate the hypothetical binding mode of the target compounds. This study provided a new molecular scaffold for the further development of anticancer as well as antimicrobial agents.

Keywords Steroid · Pyrazolone · Anticancer · Antimicrobial · Docking

Introduction

Steroids attract much attention in cell biology and pathophysiology because of the wide range of biological phenomena in which they are involved (Vejux and Lizard, 2009). The involvement of steroids in anticancer promotion and suppression has been known for a long time. This involvement goes far beyond the steroidal sex hormones (Salvador *et al.*, 2013). Many anticancer steroids are enzyme inhibitors, such as aromatase and sulfatase inhibitors for breast cancer, 5 α-reductase inhibitors for the treatment of benign prostatic hyperplasia and CYP 17 inhibitors for advanced prostate cancer therapy (Handratta *et al.*, 2005). A variety of steroids with unusual and interesting structures have been synthesized and evaluated for their antitumor activity (Krstic'a *et al.*, 2007; Poza *et al.*, 2007; Bansal and Guleria, 2008; Koutsourea *et al.*, 2008; Thibeault *et al.*, 2008). Among these steroids, nitrogen containing steroid derivatives have been shown to be more potent and have been used clinically for the treatment of cancer (Guarna *et al.*, 1999; Ling *et al.*, 1997). Among all the numerous antibiotics developed to date, few compounds possessing a steroid nucleus have been studied and the results clearly showed that these compounds exhibited a good antibacterial activity against several human pathogenic bacteria (Jayasinghe *et al.*, 1998; Atta *et al.*, 1998). The potent mechanism of action of these compounds described by the interactions of amine groups with the negative phosphate groups of LPS displacing divalent cations such as Ca^{2+} and Mg^{2+} (Nikaido, 1996; Vaara, 1993). Pyrazolones are important structural cores in many drug substances of medicinal fields. Heterocyclic nucleus containing pyrazolones are useful antipyretic and analgesic drugs (Himly *et al.*, 2003), whilst edaravone (MCI-186) has been used for treating the brain (Kawai *et al.*, 1997) and myocardial ischemia (Wu *et al.*, 2002). In addition, pyrazolones possess kinase inhibitory properties, particularly of enzymes which catalyze the phosphorylation of serine and threonine in proteins and also used for

Electronic supplementary material The online version of this article (doi:10.1007/s00044-013-0636-y) contains supplementary material, which is available to authorized users.

Shamsuzzaman (✉) · A. Mashrai · A. M. Dar · H. Khanam
Department of Chemistry Aligarh Muslim University, Aligarh 202 002, India
e-mail: shamsuzzaman9@gmail.com

A. Ahmad · M. Danishuddin · A. U. Khan
Interdisciplinary Unit of Biotechnology Aligarh Muslim University, Aligarh 202 002, India

Published online: 31 May 2013

ARTICLE IN PRESS

Journal of Saudi Chemical Society (2012) xxx, xxx–xxx

King Saud University

Journal of Saudi Chemical Society

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Synthesis, characterization, antimicrobial and anticancer studies of new steroidal pyrazolines

Shamsuzzaman [a,*], Hena Khanam [a], Ayaz Mahmood Dar [a], Nazish Siddiqui [b], Sumbul Rehman [b]

[a] *Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India*
[b] *Department of Illmul Advia, Ajmal Khan Tibbiya College, Aligarh Muslim University, Aligarh 202002, India*

Received 9 March 2012; accepted 12 May 2012

KEYWORDS

Cholest-5-en-7-one;
2,4-Dinitrophenylhydrazine;
Pyrazoline;
Antimicrobial;
Anticancer

Abstract A convenient synthesis of 2′-(2″,4″-dinitrophenyl)-5α-cholestano [5,7-*c d*] pyrazolines 4–6 from cholest-5-en-7-one 1–3 was performed and structural assignment of the products was confirmed on the basis of IR, ^{1}H NMR, ^{13}C NMR, MS and analytical data. The synthesized compounds were screened for *in vitro* antimicrobial activity against different strains during which compound 6 showed potent antimicrobial behaviour against *Corynebacterium xerosis* and *Staphylococcus epidermidis*. The synthesized compounds were also screened for *in vitro* anticancer activity against human cancer cell lines during which compound 5 exhibited significant anticancer activity.

© 2012 King Saud University. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

In the last few decades there has been an extensive focus of research towards the rational modification of steroid molecules. This is due to the fact that such type of compounds are less toxic, less vulnerable to multi-drug resistance (MDR) and highly bioavailable because of being capable of penetrating the cell wall. Recent studies reveal that incorporation of heteroatom (N/O/S) enhances the biological activities of steroid molecules. This is proved by various activities shown by these systems like antimicrobial, anti-inflammatory, hypotensive, hypocholesterolemic and diuretic activities (Manson et al., 1963; Hirschmann et al., 1963, 1964; Wang et al., 1993; Gupta et al., 1996). As a result, a number of different heterocyclic systems have been introduced into the core structure of steroids with pyrazoles, pyrazolines, isoxazoles, isoxazolines, thiazoles, thiadiazoles, pyridines, pyrimidines, imidazoles, etc. as the notable ones. Among these heterocycles, pyrazolines occupy a unique place in the realm of natural and synthetic organic chemistry (Jung et al., 2005).

Pyrazoline derivatives are synthetic targets of utmost importance for the researchers, since such type of compounds have a wide range of biological and pharmaceutical properties such as analgesic, antipyretic and antiandrogenic activities (Jung et al., 2005; Amr et al., 2005). Pyrazolines also possess antidepressant, anti-inflammatory and antirheumatic activities (Palaska et al., 2001; Bansal et al., 2001). Besides this pyrazolines are also used as potent antidiabetic agents (Villhauer et al., 2002; Ahn et al., 2004). Recently, pyrazolines were reported as a DP-IV inhibitors and antitumor agents (Amr, 2000; Hammam et al., 2000; 2003).

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

1319-6103 © 2012 King Saud University. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jscs.2012.05.004

Please cite this article in press as: Shamsuzzaman et al., Synthesis, characterization, antimicrobial and anticancer studies of new steroidal pyrazolines. Journal of Saudi Chemical Society (2012), http://dx.doi.org/10.1016/j.jscs.2012.05.004

Journal of Saudi Chemical Society (2012) xxx, xxx–xxx

King Saud University

Journal of Saudi Chemical Society

www.ksu.edu.sa
www.sciencedirect.com

ORIGINAL ARTICLE

Synthesis, characterization and anticancer studies of new steroidal oxadiazole, pyrrole and pyrazole derivatives

Shamsuzzaman *, Tabassum Siddiqui, Mohd Gulfam Alam, Ayaz Mahmood Dar

Department of Chemistry, Aligarh Muslim University, Aligarh 202 002, India

Received 2 February 2012; accepted 27 April 2012

KEYWORDS

Hydrazide;
Oxadiazole;
Pyrrole;
Pyrazole;
Anticancer;
MTT assay

Abstract In the present study steroidal derivatives, 3β-[5′-mercapto-1′,3′,4′-oxadiazole-2-yl]methoxy cholest-5-ene **2**, 3β-[2′,5′-dimethylpyrrole-1-yl]aminocarbonylmethoxycholest-5-ene **3** and 3β-[3′,5′-dimethyl pyrazole-1-yl]carbonylmethoxycholest-5-ene **4** have been synthesized from cholest-5-en-3β-*O*-acetyl hydrazide **1** using CS_2/KOH, acetonyl acetone and acetyl acetone, respectively as reagents and are characterized by IR, ^{1}H NMR, ^{13}C NMR, MS and elemental analysis. Compounds **2–4** were also evaluated for anticancer activity against human leukemia cell line (HL-60) by MTT assay and compound **4** displayed the promising behavior by showing better anticancer activity.

© 2012 King Saud University. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

1,3,4-Oxadiazoles, pyrroles, pyrazoles and their derivatives represent an important class of heterocyclic compounds with broad spectrum of biological activity. 1,3,4-Oxadiazoles have been reported to possess insecticidal (Zheng et al., 2003), herbicidal (Chavan et al., 2006), antibacterial (Shivarama Hollaa et al., 2000), antifungal (Liu et al., 2008), analgesic (Narayana et al., 2005), anti-inflammatory (Koksal et al., 2008), antimalarial (Zareef et al., 2007a), antiviral (Farghaly and El-Kashef, 2006), anti-HBV (El-Essawy et al., 2007), antianxiety (Amr et al., 2008), anticancer (Kumar et al., 2008), anti-HIV (Zareef et al., 2007b), antitubercular (Macaev et al., 2005) and anticonvulsant activities (Zarghi et al., 2005). Substituted pyrroles have been used as intermediates in the synthesis of mitomycin antitumor antibiotics possessing antibacterial activity (De Leon and Ganem, 1997; Gilchrist, 1998; Luly and Rapoport, 1983). Functionalized pyrroles are building blocks of natural tetrapyrrole pigments (Dutton et al., 1983) such as porphobilinogen or bilirubin and various other natural products and their analogs (Shen et al., 1993). Pyrazoles and their derivatives are of much importance on account of their use in therapy in different diseases (El-Hawash et al., 2006; Salgin-Goksen et al., 2007; Savelli and Alessandro, 1996). They are also reported to possess antibacterial (Abdallah et al., 2005), fungicidal (Devasia et al., 2006), antidiuretic (Vicini et al., 2002), anticancer (Rostom, 2006; Ibrahim et al., 2007), anti-HIV (Rida et al., 2005; Salih, 2008), antitumor (Salgin-Goksen et al., 2007), analgesic-anti-inflammatory (Gulcan et al., 2003; Onkol et al., 2008) and anticonvulsant (Onkol et al., 2004) properties. This gave a great impetus to the search for potential pharmacologically active drugs carrying such moieties.

Taking into consideration the existing cancer therapies, chemotherapy has turned out to be one of the most significant treatments in cancer management (Harrison et al., 2009). The natural product based drugs, Paclitaxel and Docetaxel, are extensively used in the treatment of a wide variety of cancers

* Corresponding author. Tel.: +91 9411003465.
E-mail address: shamsuzzaman9@gmail.com (Shamsuzzaman).

Peer review under responsibility of King Saud University.

ELSEVIER Production and hosting by Elsevier

1319-6103 © 2012 King Saud University. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jscs.2012.04.009

Please cite this article in press as: Shamsuzzaman, et al., Synthesis, characterization and anticancer studies of new steroidal oxadiazole, pyrrole and pyrazole derivatives. Journal of Saudi Chemical Society (2012), http://dx.doi.org/10.1016/j.jscs.2012.04.009

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2013, Article ID 436352, 8 pages
http://dx.doi.org/10.1155/2013/436352

Research Article

Synthesis, Characterization, and *In Vitro* Anticancer Activity of Newly Synthesized Steroidal 6, 7-Fused Thiazoles

Shams Uzzaman,[1] Ayaz Mahmood Dar,[1] Hena Khanam,[1] and Manzoor Ahmad Gatoo[2]

[1] *Department of Chemistry, Aligarh Muslim University, Aligarh 202002, India*
[2] *Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh 202002, India*

Correspondence should be addressed to Shams Uzzaman; shamsuzzaman9@gmail.com

Received 8 July 2012; Revised 30 October 2012; Accepted 19 November 2012

Academic Editor: Xinyong Liu

Copyright © 2013 Shams Uzzaman et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

A preparation of new series of 2′-hydrazinocholest-6-eno [4, 5-d] thiazoles **4–6** from 5α-cholestan-6-one **1–3** is herein reported. After characterization by IR, ^{1}H NMR, ^{13}C NMR, MS, and analytical data, the synthesized compounds **4–6** were tested for anticancer activity *in vitro* against the human cancer cell lines A549, HepG2, HeLa, SW480, and HL-60 by MTT as well as sulforhodamine B assay during which compounds **4–6** showed significant anticancer behaviour. The gel electrophoresis pattern demonstrated that the compound **4** alone or in presence of Cu (II) causes the nicking of supercoiled pBR322. Further the compound **4** is also able to generate reactive oxygen species (hydroxyl radical) in a dose-dependent manner, which correlates its ability to cause DNA breakage.

1. Introduction

Steroids have always attracted considerable attention because of being a fundamental class of biologically signalling molecules. In addition to their profound physiological and clinical importance [1], they have the potential to be developed as drugs for the treatment of a large number of diseases including cardiovascular, autoimmune, and brain tumours, breast cancer, prostate cancer, and osteoarthritis [2–4]. Most of the steroid-based pharmaceuticals are semisynthetic compounds prepared by connecting a special functionality to the core structure of a steroid [5]. Most important of such functionalities are the heterocyclic systems because of their potent receptor binding properties. The advantage of employing hydrophobic steroid units is their ability to interact with cell membranes and thus pave the way for biological activity of such hybrid molecules [4].

Thiazoles and their derivatives have attracted continuing interest over the years because of their varied biological activities. They have been reported as antiallergic [6], antihypertensive [7], anti-inflammatory [8], antischizophrenic [9], antibacterial [10], anti-HIV [11], hypnotics [12], and selective COX-2 inhibitors [13], fibrinogen receptor antagonists with antithrombotic activity [14], and new inhibitors of bacterial DNA gyrase B [15]. The substituted thiazoles have a number of other characteristic pharmacological features such as relative stability and ease of starting materials built in biocidal unit, enhanced lipid solubility with hydrophilicity, and easy metabolism of compounds [16].

In view of the pharmacological importance of heterosteroids particularly thiazoles and in continuation of our previous work on developing new bioactive modified steroids with heterocyclic moiety attached at ring B of tetracyclic core [17–19], our aim here is to synthesize the new steroid derivatives with a substituted thiazole ring attached at ring B of tetracyclic core and to study their *in vitro* anticancer activity.

2. Experimental

2.1. Materials and Instruments. All the reagents and solvents were obtained from the best known commercial sources and were freshly distilled. Melting points were determined on a Kofler apparatus and are uncorrected. The IR spectra were recorded on KBr pellets with Pye Unicam SP3-100 spectrophotometer and values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were run in $CDCl_3$ on a JEOL Eclipse